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SURGICAL MAGGOTS IN THE TREATMENT OF INFECTED WOUNDS: RECENT APPARATUS AND METHODS IN MAGGOT PRODUCTION AND RESEARCH*

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A PREVIOUS article in this JOURNAL[3] and a recent circular of the U. S. Bureau of Entomology[4] describe the biology of the blow flies and the production of sterile maggots. The present discussion deals with some recent devices and methods which have been found useful in maggot culture and research.

Culture Bottles for Sterile Maggots.—Aseptic rearing of maggots is carried on best in small lots. The principal reason for this is that if contamination is detected the loss in discarding that lot is correspondingly less when a small culture is used. Lots containing from 700 to 1,000 seem to be preferable.

For a culture of that size a wide mouth specimen bottle, Fig. 1 *B*, makes a simple and useful container. Shoulders are almost absent in this type of bottle which facilitates the removal of the maggots and food at the time of implantation. This is a stock bottle 3 inches high, 1½ inches wide with a mouth opening of 1¼ inches. It is made of heavy glass and is quite inexpensive. Shell vials of similar dimensions have been found useful also, but as they do not appear to be carried in stock by supply houses they have to be made up by a glass blower. Satisfactory results have also been obtained with the Naples staining jar, Fig. 1 *A*. It is made of heavy glass and has a base which prevents

*From the Division of Insects Affecting Man and Animals, Bureau of Entomology, U. S. Department of Agriculture.

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tipping. It costs about the same as the shell vial, both of which are more expensive than the stock wide mouth bottle.*

When being prepared for use, the containers are plugged with gauze-covered cotton and sterilized in a hot-air oven at 150-160° C. for one hour. The food (described later) is then added and autoclaved. Wire letter baskets are convenient for holding the containers.

Rearing Cage for Brood Larvae.—To replace fly colonies when egg production begins to decline, a certain proportion of maggots must be reared through to the adult stage. This process is somewhat different from production of sterile maggots and requires a different container. The biology involved in maggot rearing is discussed in the earlier article.[3] By far the most satis-

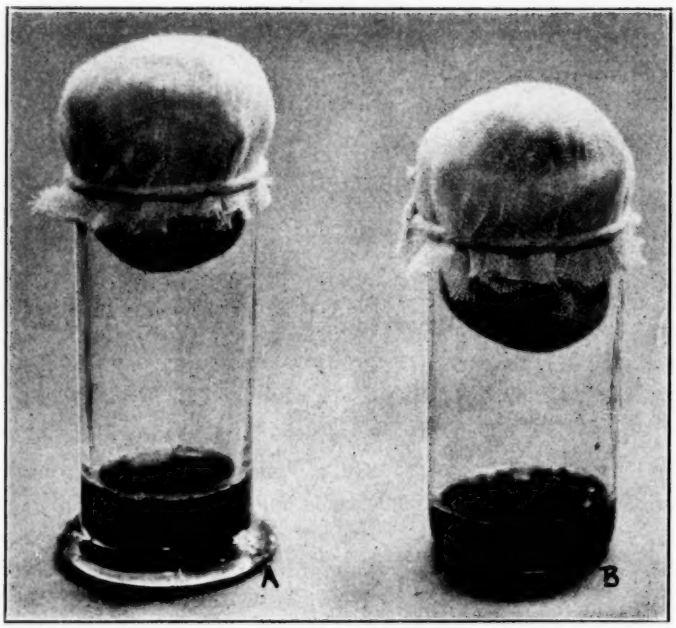

Fig. 1.—Food containers for culture of sterile maggots: *A*, Naples jar; *B*, specimen bottle.

factory cage for brood larvae, which this laboratory has used, is the double container illustrated in Fig. 2. It consists of an outer cage enclosing a food container and sand for pupation. It is a very simple device and easily obtainable. The outer cage may be a large glass beaker, a dressing jar, or preferably a commercial oiled-fiber can, as shown in the illustration, one-half gallon size, 5½ inches high and 5½ inches in diameter. The fiber cans are quite inexpensive and are useful for other purposes in the laboratory.

When the cage is being prepared for use, the material used for the migrating larvae (discussed later) is placed in the outer container. The inner one is then introduced. This may be similar to the outer one, but of much smaller size. If the fiber can is used, the inner one may be either a half-pint or pint size, preferably the former. With the meat and eggs added, this container is

*In case of uncertainty as to where to purchase these or other special articles mentioned, the Bureau of Entomology will, upon request, supply the necessary information.

placed within the cage and left uncovered. The outer cage, however, should be covered, to exclude parasites, which may become troublesome. Gases form during larval feeding and, if allowed to accumulate within the cage, tend to cause premature migration. Some ventilation is therefore necessary. A layer of cheesecloth makes a suitable covering, or, if observation is desired, perforated cellophane will be found convenient. If the fiber can is used, the cover may easily be attached by pressing the circular top out of the lid and using the rim as a collar to keep the cloth or cellophane in place.

With the closed container as described, the rearing of larvae for propagation requires but little attention. It should be noted, however, that an abundance of meat is necessary. Undernourished larvae will pupate but will develop into small flies with poor egg-laying capacity. The larvae will feed until full grown and then migrate without further care. Because of their compactness, these cages occupy little space within the incubator.

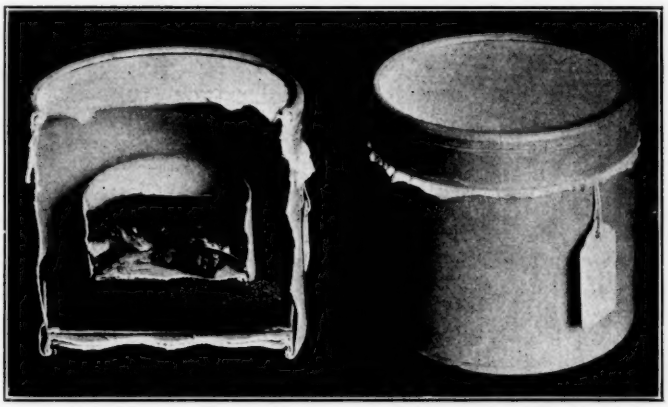

Fig. 2.—Rearing cage for brood larvae. One sectioned to show construction, and the other ready for use.

Material for Pupation of Larvae.—When the larvae begin migrating from their food, they must be provided with a suitable place for pupation. When this is supplied the maggots will enter at once; otherwise they will wander and possibly escape. Various materials, such as sand, shavings, sawdust, ground cork, and the folds of cheesecloth, have been used; but fine, clean sand is recommended by this laboratory. When sand is used it is very easy to recover pupae, when desired, by sifting. It is also available at most laboratories. About one inch of sand placed in the bottom of the outer container is sufficient.

The Water Fountain.—Where honey-yeast mixture is used as food for the adult flies, it will be noted that the mixture tends to dry out over hot weekends. Granulated sugar is an excellent substitute at such times; but when used, a supply of water must be provided. To avoid drowning of the flies, water is best supplied by means of a fountain. Several kinds have been tried, but the most satisfactory one is the type described by Miller.[2] It consists of a 50 c.c. beaker placed inverted upon a layer of filter paper in a Petri dish. The

lip of the beaker ensures a sufficient flow of water to keep the paper moist. Such a fountain will provide enough water for a cage of 150 flies for three or four days.

Determination of Number of Eggs.—In research in the field of maggot production it is sometimes necessary to know the number of eggs laid or to isolate a definite number. The eggs are usually laid in masses and remain attached to one another because of their mucoid surface. Estimating the number by the size of the clump has been found to be unreliable; on the other hand, counting individual eggs after separation is very tedious.

A method of determining the number by weight has been found satisfactory. A standard or constant was obtained by taking numerous egg-masses periodically from different colonies, weighing them, and separating and counting the eggs. In this way it was found that on the average 9.9 eggs weigh one milligram. This average may vary slightly from time to time and should be checked.

To make a determination, therefore, of the number of eggs in any clump it is necessary only to weigh the mass accurately and multiply the number of milligrams by the constant. An analytical balance should be used, preferably one of the modern type which permits rapid adjustment in milligrams. The eggs may be conveniently weighed upon a glass or tin foil plate.

Egg Viability Tests.—In the production of sterile maggots it will be found that a certain proportion of the eggs frequently fails to hatch. While this may not be important in routine maggot production, it is of significance in research, and determination of the percentage of failure under various conditions is sometimes necessary. The following method of determining viability has given good results.

Sterilized larval food is used in the test because the formation of molds or colonies of bacteria over the eggs prevents hatching. A few cubic centimeters of 1 per cent solution of plain agar in water is poured into the Naples jars or shell vials previously described. Small pieces of raw liver are then dropped into the agar. The pulverized liver mixture of Buchman and Blair[1] also gives good results. The containers are then plugged and autoclaved. The eggs used in the viability tests cannot be sterilized, as that would introduce another factor affecting hatch. A known number of eggs, probably 200 to 300, are placed without injury upon two thicknesses of wet sterile absorbent gauze about 2 inches square and transferred aseptically to the inside of the jar just above the food. Excess water should be previously drained off, otherwise the water contaminated by the unsterilized eggs might inoculate the food. The cultures are then placed in the incubator for hatching of the eggs.

When the larvae have fed for three days the food is usually sufficiently liquefied, and the maggots are large enough to strain out. Water is then poured into the jars and left for a few minutes, immersion separating the larvae rapidly from the food. If the maggots are numerous and active the water should be cold to reduce their activity while being counted. If their number is small, the food will not be sufficiently liquefied and warm water is preferable. The contents of the jars are then stirred, strained through a fine

wire sieve over a beaker, and transferred to a Petri dish. Estimation of number has been found especially unreliable, and determination of number by actual count appears essential. A hand counter has proved very useful in making counts.

Elimination of Odor.—Disagreeable odors develop readily during maggot production and research, but if sufficient care is taken they can be prevented from becoming noticeable in the laboratory. The exhaust draft recommended for the larval cabinet[3] will prevent leakage of odors into the room. The ordinary type of household fan, however, placed within the cabinet to force air up a ventilator is usually not satisfactory. An exhaust fan installed on the ventilator line to draw off the odors is more preferable and is not expensive.

Residue from the feeding of brood larvae is especially offensive and should be promptly destroyed. Each laboratory will probably have certain facilities for waste removal. A very satisfactory method is to use a paper bag in a covered receptacle such as a large dressing jar or a can and to keep this in the larval cabinet or in a chemical hood. At the end of each day the bag is burned. An incinerator or steam generating plant is usually available to the laboratory.

REFERENCES

1. Buchman, J., and Blair, J. E.: Maggots and Their Use in the Treatment of Chronic Osteomyelitis, Surg. Gynec. Obst. **55**: 177, 1932.
2. Miller, D. F., Doan, C. A., and Wilson, E. H.: The Treatment of Osteomyelitis (Infection of Bone) With Fly Larvae, Ohio J. Sc. **32**: 1, 1932.
3. Robinson, Wm.: Surgical Maggots in the Treatment of Infected Wounds: Culture of Sterile Maggots, J. LAB. & CLIN. MED. **18**: 406, 1933.
4. Robinson, Wm.: The Culture of Sterile Maggots for Use in the Treatment of Osteomyelitis and Other Suppurative Infections, Circular E-311, Bureau of Entomology, U. S. Dept. Agr., 1933.

HYPOLEUCOCYTIC ANGINA*

An Unusual Form of Infectious Leucopenia

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THIS syndrome is clinically similar to agranulocytic angina in its sudden onset, high fever, prostration, and the presence of necrotic ulcerations of the mucous membranes, particularly in the oropharynx. The blood count, however, differs from that usually found in agranulocytic angina, as well as from other forms of leucopenia, in that there is in addition to the profound leucopenia a relative polynucleosis of the nonsegmented neutrophiles. In all cases of agranulocytic angina reported in the literature, the leucopenia has been the result of a marked diminution or absence of the polymorphonuclear neutrophiles, which in fatal cases usually have disappeared completely from the circulating blood as well as from the bone marrow (Schultz[1]).

Ulcerations of the mucous membranes and an extreme leucopenia may occasionally be present in other conditions simulating agranulocytic angina, notably in leucopenic leucemia, aplastic anemia, benzol and arsphenamine poisoning, and poisoning from radium or x-rays. In these conditions, however, marked alterations are also found in the hemoglobin, red blood cells or platelets, and at times immature myeloid cells are present in the peripheral blood. Ulcerations in the oropharynx, and rarely leucopenia may likewise occur at the onset of infectious mononucleosis (McKinlay and Downey[2]).

The three cases to be reported in this paper, although clinically resembling agranulocytic angina, showed a relative polynucleosis (nonsegmented neutrophiles), associated with a profound leucopenia, with a more or less parallel reduction in the lymphocytes and monocytes. The red blood cells and platelets remained unaffected.

Detailed reports of these cases follow:

Case 1.—I. K., female, aged fifty-two, married; admitted to private pavilion of Mount Sinai Hospital (Service of Dr. A. A. Epstein), December 19, 1928.

Previous History.—Following an operation for fibroids eleven years previously, the patient developed persistent and irregular pain in the epigastrium, which became severe while eating, but subsided somewhat about two hours after the ingestion of food. From time to time there was considerable nausea, but no vomiting. The appetite was poor; the diet consisted chiefly of crackers and tea. The bowels were constipated. The patient entered the hospital for an exploratory laparotomy.

Examination on Admission.—The patient was well developed. The mouth, throat, heart, and lungs were normal. The liver and spleen were not palpable. There was slight tenderness in the epigastrium. The urine showed a faint trace of albumin and occasional hyaline casts.

*From the Wards and Laboratories of the Mount Sinai Hospital.
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The blood examination showed a slight leucopenia:

Hemoglobin	85 per cent
Red blood cells	4,000,000
White blood cells	4,500
Polymorphonuclear neutrophiles	55 per cent
Lymphocytes	45 per cent

Course and Treatment.—An exploratory laparotomy was performed by Dr. A. A. Berg on Dec. 26, 1928. A diverticulum on the posterior wall of the duodenum was found. Otherwise, no other abnormality was noted. Following the operation, the temperature rose to 101.4° F. and remained around that level for two weeks.

About one week after the operation (Jan. 2, 1929), patient complained of a sore tongue and considerable pain on swallowing. Extensive ulcerations were found not only on the tongue, but also on the tonsils, uvula, pillars of the fauces, and on the hard and soft palates.

Fig. 1.—Nonsegmented neutrophiles in the peripheral blood showing "toxic" granules in the cytoplasm.

These were covered with yellowish membranes, easily removed without bleeding. Cultures of the ulcerations were negative for diphtheria bacilli and smears showed numerous streptococci and bacilli; and few spirilli. The patient became progressively weaker, as the condition of the mouth and throat did not respond to local treatment. Transfusion (500 c.c.) was followed by temporary improvement.

Three days later (Jan. 5, 1929), as the prostration, fever (104° F.), and oropharyngeal necroses became pronounced, the clinical symptoms suggested agranulocytic angina. The blood picture was compatible with this diagnosis, with the exception of the differential count. There was a relative increase in the percentage of the nonsegmented neutrophiles (the staff and young cells of the Schilling hemogram), associated with a profound leucopenia (Table I).

The neutrophiles, both segmented and nonsegmented, presented severe toxic changes (Fig. 1) in the cytoplasm. These consisted of the appearances of coarse granules and vacuolization. All the neutrophiles in this case showed such toxic changes as well as anisocytosis and poikilocytosis.

These abnormal blood changes persisted (Table I).

TABLE I

CASE 1

	JAN. 5, 1929	JAN. 6, 1929	JAN. 7, 1929 BEFORE RADIOTHERAPY	JAN. 7, 1929 6 HOURS AFTER RADIOTHERAPY
Hemoglobin	70%	74%	70%	
Red blood cells	3,400,000	3,800,000	4,000,000	
White blood cells	2,200	900	1,000	1,600
Platelets	450,000	430,000	240,000	
Polymorphonuclear nonsegmented cells	52%	65%	44%	57%
Polymorphonuclear segmented cells	5%	2%	5%	8%
Lymphocytes	35%	26%	40%	29%
Monocytes	4%	4%	7%	4%
Plasma cells				1%
Macrophages			1%	
Myelocytes neutrophilic	4%	2%	3%	
Degenerative index	100%	100%	100%	100%

Comment.—The severe leucopenia associated with a high percentage of nonsegmented neutrophiles in itself was an unfavorable prognostic sign. The number of neutrophiles with toxic granules in the cytoplasm (Fig. 1) was very high (100 per cent). Such cytoplasmic toxic degeneration has been previously noted by Gloor,[3] Mommsen and others.[4] The quantitative measure of these pathologic neutrophiles is obtained by dividing their number by the total number of neutrophiles. This has been previously designated by us[5] as the *degenerative index.* The persistence of a degenerative index of 100 usually indicates an unfavorable prognosis. The rapid increase of the degenerative index is usually associated with an unfavorable outcome. The index, therefore, may be regarded as a valuable aid in prognosis.

CASE 2.—R. C., female, aged sixty-two, admitted to Mount Sinai Hospital (Medical service of Dr. George Baehr), July 8, 1930, as a case of agranulocytic angina.

Previous History.—For six years preceding, the patient's complaints were chiefly cardiac, dyspnea, palpitation, and precordial pains.

Present Illness.—Six days previous to admission, there was generalized abdominal pain and diarrhea, followed by fever, progressive weakness, angina, and increasing dysphagia. Four days previously, swelling of the neck and parotid regions developed.

TABLE II

CASE 2

	JULY 8, 1930	JULY 9, 1930	JULY 10, 1930	JULY 11, 1930	JULY 12, 1930	JULY 13, 1930
White blood cells	1,800	2,200	11,600	10,000	9,600	19,900
Polymorphonuclear nonsegmented cells	39%	44%	54%	50%	50%	60%
Polymorphonuclear segmented cells	10%	13%	11%	30%	39%	12%
Lymphocytes	33%	30%	21%	6%	9%	16%
Monocytes	13%	1%	12%	10%	2%	11%
Myelocytes	5%	6%	2%	4%		1%
Degenerative index	100%	100%	100%	100%	100%	100%

Examination on Admission.—The patient was obese, flushed, toxic in appearance, and somewhat stuporous. Several lesions resembling pustules were present on the skin. The mucous membrane of the mouth was dry, glazed and reddened; a large necrotic black slough was adherent to the palate and extended to the pillars of the fauces and the posterior pharyngeal wall. Tender swellings were found in both submaxillary regions and over the

left parotid glands. There was slight rigidity of the neck. Heart sounds were irregular and of poor quality. Breathing was stertorous.

Course and Treatment.—The clinical course was suggestive of agranulocytic angina. The blood picture, however, showed a white cell count of 1,300 with 49 per cent polymorphonuclear neutrophiles, and 39 per cent nonsegmented cells (Table II); the hemoglobin, red blood cells, and platelets were normal. Blood cultures, taken on two occasions, were negative. All of the neutrophiles contained toxic granules (degenerative index 100).

Signs of myocardial failure developed and on two occasions pulmonary edema, which subsided with medication (atropine and adrenalin). There was a transitory improvement for a few days after a blood transfusion (500 c.c.). The ulcerations and leucopenia both subsided. The white cells increased rapidly (two days) to 11,600, with 65 per cent polymorphonuclear neutrophiles when the patient developed a right suppurative parotitis. Following this, the condition became considerably worse with signs of pneumonia and a rise in temperature to 105.8° F. and death ensued.

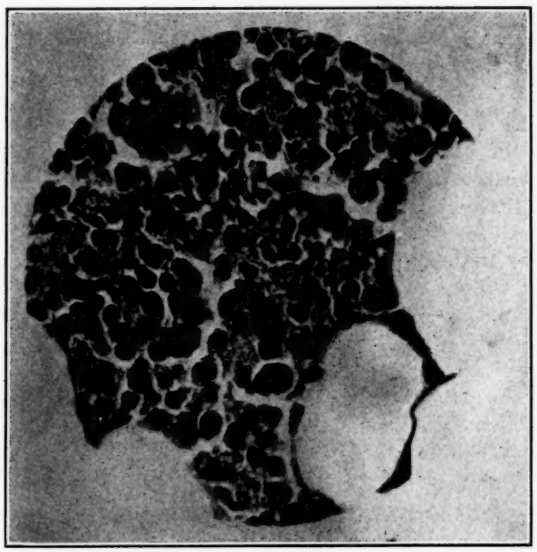

Fig. 2.—Bone marrow of Case 2. Note the hyperplastic bone marrow with many mature and immature myeloid cells

Comment.—In this case the rise of the white blood cells from 1,800 to 10,000 after two days in the hospital would ordinarily be considered a favorable sign. In following the blood picture daily (Table II), two signs were significant: (1) the nonsegmented neutrophiles steadily rose from 39 per cent to 60 per cent; and (2) the degenerative index persisted at 100 per cent throughout the course of the disease. Although some of the hematologic findings were suggestive of apparent recovery, the severe complications (parotitis and pneumonia) led to a fatal outcome as was prognosticated by other blood changes, i.e., the high degenerative index, and the progressive increase of the nonsegmented neutrophiles.

CASE 3.—J. J., male, aged thirty-five, physician, admitted to Mount Sinai Hospital (Surgical service of Dr. Neuhof and Otolaryngological Service of Dr. Yankauer), on Nov. 5, 1930.

Previous History.—Negative.

Present Illness.—Three days previous to admission (Nov. 2, 1930) patient had been admitted to Willard Parker Hospital, with a suspected laryngeal diphtheria following an attack of respiratory difficulty accompanied by stridor and pain in the chest. Had also complained of dysphagia and hoarseness, and an acute pharyngitis and laryngitis, and a membranous pharyngeal ulceration extending to the larynx were found. Temperature was

102° F., and later 104° F. The throat culture showed *Streptococcus hemolyticus.* The blood picture was apparently normal as the white blood cells were 6,500 with 63 per cent polymorphonuclear neutrophiles. Under observation, the patient's condition became worse, with more marked laryngeal involvement. Within a few days the white blood cells diminished to 2,500 without any change in the percentage of polymorphonuclear neutrophiles. He received two blood transfusions.

On admission to the Mount Sinai Hospital, the Wassermann reaction was negative. The blood examination showed a profound leucopenia with a marked increase of the nonsegmented neutrophiles and was as follows:

Hemoglobin	85 per cent
Red blood cells	4,800,000
White blood cells	3,500
Polymorphonuclear nonsegmented cells	65 per cent
Polymorphonuclear segmented cells	10 per cent
Lymphocytes	25 per cent
Degenerative index	88 per cent

Course and Treatment.—The stridor became more marked and the patient experienced a great deal of difficulty in breathing.

A tracheotomy was performed for suspected perilaryngeal phlegmon. The tissue was found to be edematous but no pus was present. Following the operation the temperature gradually returned to normal and the stridor appreciably decreased. Cultures from the region of the laryngeal slough revealed numerous colonies of *Streptococcus hemolyticus.* Sections made from the necrotic tissue removed from the larynx showed necrotic purulent exudate with numerous bacterial colonies. The necrotic lesions in the pharynx healed rapidly. Progress following the tracheotomy was rather slow. The condition of the larynx was considered a necrotizing process as a result of a severe streptococcic infection. The entire right side of the larynx down to the true vocal cords sloughed away. For a time this caused marked discomfort as the loss of structure resulted in aspiration of fluids on swallowing. For a few weeks, nasal feeding was necessary. The patient was also given several transfusions and frequent intravenous glucose infusions. Save for a slight pneumonic infiltration noted only on x-ray examination (Nov. 8, 1930), no complications developed. The temperature became normal about November 10.

After the fourth week in the hospital, the slough in the larynx entirely separated and healthy granulations could be seen. On examination, the distortion and the granulations had a peculiar appearance resembling a neoplastic condition. The patient's voice and general condition improved. When the laryngeal condition was almost healed, about the middle of December, a permanent tracheotomy tube with a safety valve was inserted. Since discharge from the hospital (on Dec. 12, 1930), the patient is apparently well save for the granulomatous condition in the larynx.*

The blood picture (Table II) during the course of the disease is of singular interest. The white blood cells increased to normal by Nov. 13, 1930; however, the nonsegmented cells remained increased during the entire period of observation at the hospital. The degenerative index, which gradually increased the first few days at the hospital, returned to normal about November 24.

DISCUSSION

Three cases of severe infection associated with necrotic lesions of the mucous membranes and an unusual type of leucopenia have been described.

Clinically these cases resembled agranulocytosis. The onset of hypoleucocytic angina was apparently gradual and insidious, about six days elapsing before it fully developed. The first symptom noted was soreness of the throat or tongue, associated with dysphagia. Hoarseness and marked stridor were present

*This patient died on Nov. 17, 1933, after several attacks of true agranulocytosis, which began in May, 1933.

in one case. Fever was usually slight at the onset, but increased as the disease progressed, reaching 104° to 105° F. At the height of the disease, the patients were prostrated, and appeared flushed and toxic. The tongue, tonsils, pharynx, larynx, or all of them showed extensive sloughing. Large, irregular, sharply delineated necrotic areas were present. These were covered with a yellowish or blackish membrane. Throat cultures were positive for hemolytic streptococci. No diphtheria bacilli were found. In one instance, a section of necrotic slough showed a purulent exudate with numerous bacterial colonies. In two cases, the blood cultures were negative; no blood culture was made in the third.

At the height of the disease, the blood picture showed a profound leucopenia with several unique features. Although there was a marked depression in the total white cell count, the differential count retained the usual relations. The presence of a high percentage of nonsegmented polymorphonuclear neutrophiles showing degenerative changes in the cytoplasm (Fig. 1) confirmed the impression that a severe infection was present. The hemoglobin, red cell count and platelets were unaffected. In one case the bone marrow was obtained and was found to be hyperplastic with an abundance of mature and immature myeloid cells (Fig. 2). This is in striking contrast to true agranulocytic angina, in which at the height of the disease, there is ordinarily a complete disappearance of the granulocytes from the blood stream, as well as from the bone marrow.

CONCLUSION

A study of the blood picture in the three cases described in this article revealed an unusual form of leucopenia in a condition termed by the authors "hypoleucocytic angina" to describe a clinical syndrome similar to that of agranulocytic angina, but which differs hematologically from the latter as well as from other forms of leucopenia, in that there is a relative polynucleosis of the nonsegmented neutrophiles.

REFERENCES

1. Schultz, W.: Die akuten Erkrankungen der Gaumenmandeln, Berlin, Julius Springer, 1925.
2. Downey, H., and McKinlay, C. A.: Acute Lymphadenosis Compared With Acute Lymphatic Leukemia, Arch. Int. Med. **32**: 82, 1923.
3. Gloor, W.: Die klinische Bedeutung der qualitativen Veraenderungen der leukozyten, Leipzig, George Thieme, 1929.
4. Mommsen, H.: Ueber die neutrophilen granulationen der Leukocyten und ihre gesetzmaessige Veraenderungen bei Scharlach und lobaerer Pneumonie, Jahrb. f. Kinderheilk. **116**: 293, 1927.
5. Kugel, M. A., and Rosenthal, N.: Pathologic Changes Occurring in Polymorphonuclear Leukocytes During the Progress of Infections, Am. J. Med. Sc. **183**: 657, 1932.

THE GRAM PROPERTY OF THE ACID-FAST FORM OF THE TUBERCLE BACILLUS*

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IT IS stated in standard textbooks of bacteriology[1] that the tubercle bacillus is gram-positive. It seems that this statement has never been seriously challenged. This property of the tubercle bacillus is of more than mere theoretical interest, it is of definite diagnostic importance due to the claim that certain bodies, called Much "granules," are nonacid-fast gram-positive forms of a hitherto unknown tuberculosis virus which are often alleged to be found instead of the typical acid-fast tubercle bacilli within cold abscesses and in some sputa. The questions raised in this and in a succeeding paper are: (1) Does the tubercle bacillus possess the Gram staining property? (2) Is the Much "granule" stain a modified Gram stain?

DOES THE TUBERCLE BACILLUS POSSESS THE GRAM STAINING PROPERTY?

The purpose of this paper is to demonstrate that the tubercle bacillus in its pathogenic acid-fast phase does not possess the genuine Gram property; that although it is credited with being gram-positive even in the most authoritative treatises,[1] the tubercle bacillus, in its acid-fast stage, is by nature of its physicochemical constitution an organism to which the Gram staining method cannot properly be applied. It is here advisedly stated that, in its pathogenic stage the tubercle bacillus is acid-fast, or, conversely, that the nonacid-fast forms of the organism have not yet been shown to produce typical tuberculosis in the experimental animal. It is true that Much claims by means of his so-called modified Gram stain to have discovered an atypical nonacid-fast gram-positive form of the tuberculosis virus. In a succeeding paper I have taken issue with part of this contention. Sweeny[2] by means of the single isolation method of Janse-Peterfi in modified form, produced a pathologic condition by the injection of a single nonacid-fast coccoid mutation form of the tubercle bacillus. The disease so produced is not, however, a typical tuberculosis.

When aniline, as employed in the standard Gram stain, is used in combination with a basic stain such as gentian violet or basic fuchsin in staining the acid-fast form of the tubercle bacillus, it renders these dyes acid-fast similar to the action of phenol. This action of aniline is not surprising since it is phenylamine, a benzene ring compound to which class of chemical compounds belongs also phenol. Even before the Ziehl acid-fast method of staining the tubercle bacillus was devised, Ehrlich[3] employed a solution of gentian violet in aniline water for demonstrating the tubercle bacillus. He found that this stain resisted decolorization with 33⅓ per cent nitric acid when

*Received for publication, January 7, 1933.

applied to this organism. Hence, instead of the phenol of the carbol-fuchsin, the aniline served with similar efficiency in bringing about retention of the original dye; the aniline dyes were acid-fast similar to the carbol-dyes and likewise alcohol-fast, since Ehrlich's method employed 60 per cent alcohol as decolorizer after application of the acid.

Benians[4] states that the tubercle bacillus does not require iodine to enable it to withstand alcohol and acid-alcohol decolorizers. He fails to cite experimental work. The same writer says, likewise, that in presence of the acid-fast property it is impossible to fully demonstrate alcohol-fastness, i.e. in the face of acid-fastness, the Gram property cannot be fully exhibited.

EXPERIMENTAL

General Technic.—Untreated tubercle bacilli from a pure culture were applied to slides in a drop of distilled water, and dried in a paraffin oven at 55° C. for a few minutes. No fixation was applied to cause the organisms to adhere so as to prevent the introduction of artefacts.

I. COLD STAINING OF THE TUBERCLE BACILLUS WITH THE GRAM STAIN

Experiment 1.—Tubercle bacilli were stained according to the standard Gram stain but no counterstain was used. The bacilli were stained cold with aniline gentian violet (Stirling's) for one minute, Gram's iodine solution one minute, and decolorized with alcohol until color ceased to come off; no counterstain was applied.

Result: The tubercle bacilli were stained an intense deep purple color.

Experiment 2.—Tubercle bacilli were stained with formalin gentian violet, which is occasionally used in the Gram stain, cold for one minute, Gram's iodine solution one minute, decolorization with alcohol, one slide with and one without counterstain.

Results: Neither slide showed any stained tubercle bacilli.

Experiment 3.—Tubercle bacilli were stained with cold aniline fuchsin, the remainder of the procedure as in (2) above.

Result: The organisms were not stained.

Experiment 4.—Tubercle bacilli were stained with cold crystal violet as carried out by Ruhland,[5] the remainder of the procedure as in (2) above.

Result: No appreciable staining of bacilli.

Experiment 5.—Purpose: To ascertain the effect of carbolfuchsin on the tubercle bacillus when applied cold. A preparation of a slide of tubercle bacilli was stained cold with carbolfuchsin for one minute, not treated with iodine solution nor decolorized with alcohol.

Result: The bacilli were stained faintly or not at all.

Comments on Experiments 1 to 5: Carbolfuchsin, aniline fuchsin, and crystal violet do not stain the tubercle bacilli when applied cold (for one minute); aniline gentian violet, however, stains the tubercle bacilli intensely when applied cold for one minute, nor does it decolorize with alcohol. Whereas, phenol in combination with fuchsin will stain the tubercle bacilli when applied hot, it fails to stain them when applied cold; aniline, on the other hand, in combination with fuchsin fails to stain the tubercle bacillus when applied cold but when combined with gentian violet does so readily. Formalin gentian violet and crystal violet dyes that are frequently used in Gram staining fail to stain the tubercle bacillus when applied cold.

It will thus be seen that aniline fortifies only gentian violet in cold staining.

II. EFFECT OF IODINE ON THE TUBERCLE BACILLUS

a. *With Alcohol as Decolorizer.—*

Experiment 6.—"Gram" stain without iodine solution. Tubercle bacilli were stained with aniline gentian violet as in Experiment 1 but using no iodine; decolorization with alcohol.

Result: The organisms were stained as intensely as when iodine was used.

Comment on Experiment 6: Iodine is not necessary to "mordant" the aniline gentian violet as is the case with nonacid-fast organisms in order to enable the tubercle bacilli to retain this stain. Hence, one of the essential stages in the classical Gram stain—the "mordanting" with iodine, has thus been shown to be unnecessary when applied to the tubercle bacillus to enable the latter to retain the gentian violet when alcohol is employed as the decolorizing agent.

b. *With Acid-Alcohol as Decolorizer.—*

Experiment 7.—Tubercle bacilli were stained with cold aniline gentian violet for one minute, Gram's iodine solution one minute, acid alcohol (3 per cent HCl in 70 per cent alcohol) until color ceases to come off. This acid alcohol is the same as that employed in the Ziehl-Neelsen stain.

Results: Tubercle bacilli were intensely stained a dark blue color; they were not decolorized by the acid alcohol; in other words, they were acid-fast to the Gram stain.

Experiment 8.—Same as Experiment 7, but without the Gram's iodine solution.

Results: The tubercle bacilli were stained as intensely as in Experiment 6.

Experiment 9.—Living cultures of recently isolated gram-positive pathogenic staphylococci and streptococci were stained according to Gram and decolorized with acid alcohol as employed in the Ziehl-Neelsen stain. They were then counterstained with Bismarck brown.

Results: These gram-positive organisms were completely decolorized by acid alcohol and took the counterstain.

Comment on Experiments 7, 8, and 9: The tubercle bacilli will retain the aniline gentian violet as well when the organisms are not "mordanted" with Gram's iodine solution as when they are so treated, not only after alcohol but also after acid alcohol is applied as decolorizer. This is not the case with pyogenic gram-positive organisms such as staphylococci and streptococci. When these are exposed to acid alcohol even after application of iodine solution they will lose their gentian violet, which is to be expected since they are not acid-fast but only alcohol-fast (gram-positive); the tubercle bacillus, however, is alcohol and acid-fast. This constitutes, consequently, a basic deviation from the Gram stain in the case of the tubercle bacillus, so vital as to force the conclusion that the tubercle bacillus does not possess genuine Gram property since, first, it does not require iodine to remain alcohol-fast, and second, the bacillus stained with aniline gentian violet is acid-fast.

STANDARD GRAM STAIN APPLIED TO CHEMICALLY TREATED TUBERCLE BACILLI

Living tubercle bacilli from cultures were deprived of their acid-fastness by strong acid and alkali. This often is rather difficult to accomplish completely in all of the tubercle bacilli treated, if the morphology of the organisms is to be retained. The purpose of this mode of experimentation was to ascertain the reason for the atypical Gram property of the tubercle bacillus, in

which portion of the tubercle bacillus it is vested, and to determine the Gram property of the tubercle bacillus which has been deprived of its acid-fast property.

Tubercle Bacilli Treated With Sodium Hydroxide.—As Long[6] has pointed out, the acid-fastness of the tubercle bacillus is highly resistant to this alkali in rather strong solutions. In the following experiments living tubercle bacilli were dried and pulverized by passing through a fine mesh copper sieve (1,936 per sq. in.) and exposed in centrifuge tubes to a 25 per cent solution of sodium hydroxide for varying periods of time. The hydroxide was then carefully decanted so that the bacilli adhered to the wall of the tube, and the organisms washed and centrifuged several times until the washings were neutral to litmus. They were then applied without fixative to slides, placed in the paraffin oven at 55° C. for a few minutes until dry and then stained.

Experiment 10.—Exposure time to 25 per cent sodium hydroxide: five minutes.

Result: Tubercle bacilli well stained with carbolfuchsin. The granules seemed somewhat swollen. The intergranular portion of the bacilli generally is not stained with carbolfuchsin, it often remains colorless as is frequently the case in untreated bacilli.

Experiment 11.—Exposure time same as Experiment 10. The organisms were Gram stained after application of hydroxide. This shows the granules stained very intensely purple. The intergranular material in many bacilli stains brown with the counterstain (Vesuvin). Hence, we have the apparent anomaly of an organism staining "gram"-positive and "gram"-negative in the same bacillary body.

Experiment 12.—Exposure time: one and one-half hours.

Results: Ziehl-Neelsen stain showed a number of nonacid-fast tubercle bacilli which stained faintly blue with the methylene blue. Those that stained acid-fast were colored a very faint red.

Experiment 13.—Exposure time: one and one-half hours. Gram stain applied.

Results: These tubercle bacilli are stained more intensely with aniline gentian violet than with carbolfuchsin in Experiment 10. Some take the counterstain, i.e. they are colored brown by the vesuvin. These tubercle bacilli that have been rendered gram-negative, stain uniformly throughout with no granules whatever, and the bacillary body is intact.

Experiment 14.—Exposure time: four days. Practically all the tubercle bacilli have been disintegrated. However, even after this period of time a rare bacillus staining acid-fast and "gram"-positive is seen.

Experiment 15.—Tubercle bacilli not treated with sodium hydroxide were exposed to Bismarck brown, heating the stain for one minute. No stained tubercle bacilli were to be seen. Some of the extrabacillary amorphous material only stains brown. This effect of Bismarck brown on the tubercle bacillus is in keeping with the work of Hope Sherman.[7]

Comments on Experiments 10 to 15: After being exposed to sodium hydroxide the tubercle bacillus can be made to manifest an apparent anomaly of being simultaneously "gram"-positive and "gram"-negative in the same bacillus. This is explainable by assuming that the sodium hydroxide brings about changes in the intergranular portion of the bacillus facilitating penetration of the Gram counterstain, Bismarck brown, which by itself (Experiment 15), will not adequately stain the untreated tubercle bacillus. This intergranular portion of the untreated tubercle bacillus is often quite resistant to strong dyes as is evidenced by unstained areas between the granules of many of the organisms after the Ziehl-Neelsen stain. This double staining

after treatment with sodium hydroxide can be explained on the basis of Experiments 6, 7, 8, and 9, above. The aniline gentian violet of the Gram stain, as has been shown in these experiments acts as an acid-fast stain for the acid-fast portion, especially the granules of the tubercle bacillus in these treated organisms, and the investment of the nonstaining intergranular portion having been removed or altered by the hydroxide is rendered permeable to the Bismarck brown and shows genuine gram-negative property. As has been seen in Experiment 15, Bismarck brown does not appreciably stain the untreated tubercle bacillus.

Tubercle Bacilli Treated With Sulphuric Acid.—The purpose of this work was to render the tubercle bacillus nonacid-fast with sulphuric acid in a rapid and certain manner and then to study the Gram property of the nonacid-fast organism.

Experiment 16.—Dried living tubercle bacilli that were passed through a fine sieve were exposed to the action of 2.5, 7.5, 10, 15, 20, 50, and 75 per cent sulphuric acid solutions.

Results: The tubercle bacilli in the center of small clumps often remained acid-fast even in the higher percentages, after five minutes' exposure. Hence concentrated sulphuric acid was employed to deprive the organisms of their acid-fastness completely and as quickly as possible without altering their morphology.

Experiment 17.—Concentrated sulphuric acid (sp. gr. 1.84) was applied to pulverized intact tubercle bacilli. The latter floated on the surface and could, after exposure time had elapsed, be skimmed off with a coiled platinum loop and washed with distilled water into a centrifuge tube. The tubercle bacilli were then centrifuged, and repeatedly washed until the wash water was neutral to litmus. The organisms were then applied to slides, dried at room temperature, and stained. When the tubercle bacilli were exposed much longer than five minutes to concentrated sulphuric acid, they generally disintegrated and showed mostly amorphous débris which stained gram-negative. One-minute exposure showed that some of the tubercle bacilli were losing their acid-fast material which latter appeared as round or oval droplets in the optical fields. Often these were still attached to the bacillus which stained blue (nonacid-fast), and the former bright red (acid-fast) (see Plate I).

Results: Thus the acid-fast material was "extracted" by the sulphuric acid from the tubercle bacilli and the bacillus was left morphologically intact, staining gram-negative, if treatment was not too prolonged so as to produce disintegration of the bacillary bodies.

Five minutes' exposure at room temperature brought about a complete abolition of acid-fastness. The bacilli were stained uniformly throughout the bacillary body a bright blue by the contrast stain. With the Gram method of staining the same bacilli stained a brilliant brown with the vesuvin, the dye being evenly distributed throughout the bacillus and the latter free from all beading. Hence they had been rendered gram-negative by removal of the acid-fast material. The morphology and staining of these tubercle bacilli deprived of their acid-fastness were identical with the staining of nonacid-fast gram-negative young bacilli found in a culture obtained directly from sputum grown on potato medium.

Comment on Experiments 16 and 17: From these experiments it will be seen that after the acid-fast materials in the tubercle bacillus have been removed or destroyed chemically there remains a bacillus "underneath" that is gram-negative. In other words, deprived of its acid-fast material which has no genuine Gram property, the "basic portion" of the tubercle bacillus is gram-negative. This Gram counterstain colors the entire tubercle bacillus uniformly throughout with the Bismarck brown counterstain, both what was the granular and intergranular portion of the organism. These bacilli stained

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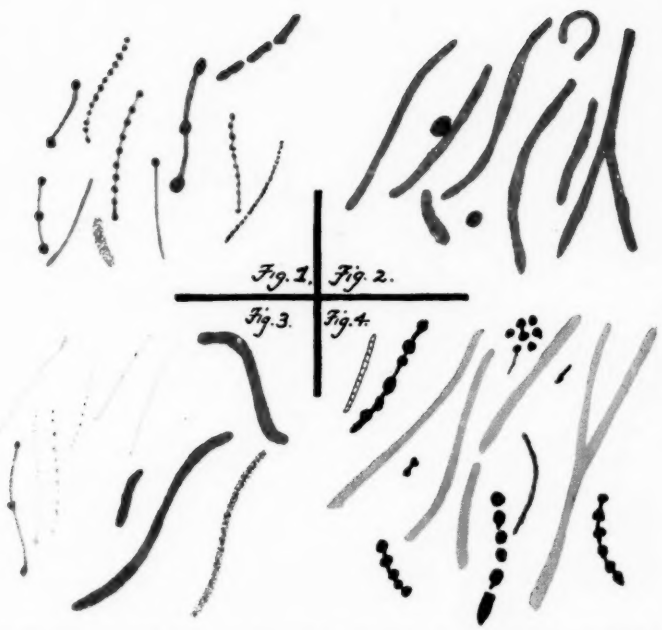

Fig. 1.—Acid-fast beaded tubercle bacilli which were treated with sulphuric acid, with the results seen in Fig. 2. Note how slender they are in comparison with nonacid-fast forms in Figs. 2 and 3.

Fig. 2.—Ziehl-Neelsen stain of tubercle bacilli after treatment with concentrated sulphuric acid for one minute. Bacilli have taken the counterstain; they are nonacid-fast. Acid-fast droplets, one of which is still clinging to a tubercle bacillus, are shown. These latter stained "gram-positive," whereas the bacilli were gram-negative. The beading has disappeared and the nonacid-fast tubercle bacilli stain uniformly and their morphology is intact. They resemble the nonacid-fast bacilli seen in Fig. 3.

Fig. 3.—Culture stained with Ziehl-Neelsen stain, showing acid-fast, some nonacid-fast and partially nonacid-fast forms. Note similarity of the morphology of the "young" nonacid-fast bacilli to those deprived of their acid-fastness by sulphuric acid shown in Fig. 2.

Fig. 4.—Gram stain (Bismarck brown counterstain) of a culture of tubercle bacilli containing some nonacid-fast tubercle bacilli shown in Fig. 3. The bacilli are heavy rods and stain evenly without beading.

with the counterstain possess none of the aberrant morphology of the untreated acid-fast tubercle bacilli, but are uniform rods of varying lengths, with rounded ends. Moreover, the tubercle bacillus may be violently deprived of its acid-fastness and of its so-called gram-positivity and yet retain its morphology, that is, not to be disintegrated. Benians[4] found that mechanical disruption of the body of the tubercle bacillus destroyed both its acid-fastness and its "gram-positivity" and rendered the bacillary débris gram-negative. However, it will be seen from the above work that the tubercle bacillus may be deprived of its acid-fast property completely without causing disintegration of the bacillary body. The capsule of the bacillus, if it has one, following treatment with sulphuric acid seems to remain intact as the bacillus retains a definite bacillary morphology after such exposure. The bacillus so deprived of its acid-fast material appears wider than the average tubercle bacillus, has rounded ends, and stains uniformly throughout without granulations or unstained areas. The impression one receives is that the tubercle bacillus consists primarily of a gram-negative bacillus (young organism) of rather uniform staining property which in the course of its metabolism becomes encrusted externally to its investment ("capsule") or within the latter with acid-fast, lipoidal material which is frequently unevenly distributed in the form of granules. Simultaneously with this process the "capsule" becomes impervious to all dyes as is evidenced by the unstained portions of the bacillus between the granules. The presumption is that the hydroxide dissolves off this investment.

STAINING OF THE ETHER EXTRACT OF THE TUBERCLE BACILLUS

Experiment 18.—Tubercle bacilli were extracted with ether for ten months. This ether extract was placed on slides and the ether allowed to evaporate. The slides were then stained according to Ziehl-Neelsen and Gram methods.

Result: The former showed only slightly acid-fast staining property; the latter stained a deep purple.

Comment: It would appear that the lipoidal extract of the tubercle bacillus is more "gram"-positive than acid-fast, due probably to the greater resistance of aniline gentian violet than carbolfuchsin to acid alcohol and also to the greater visibility of the former. The ether extract is, like the unextracted lipoid in the tubercle bacillus, both alcohol- and acid-fast and hence has no genuine gram-positive property since the latter demands only a resistance to alcohol (alcohol-fastness).

DISCUSSION

In order to clarify the discussion it might be well to define what is meant by the Gram and by the acid-fast property. For an organism to possess the Gram property it must either lose or retain the original stain which has been "mordanted" with the special Gram's iodine solution, when alcohol or acetone are applied as decolorizers. In the former case the organism is "gram-negative," in the latter "gram-positive." An organism is acid-fast if the dye with which it has been stained fails to be removed when treated with dilute mineral acids. Incorporated with this acid-fast staining solution is some chemical

such as phenol to aid penetration. In addition it may be necessary to apply heat to bring about penetration into the microorganism. Alcohol is not an essential decolorizing ingredient in the acid-fast staining method, it being employed on the assumption that the tubercle bacillus is more resistant to it than are certain other acid-fast organisms such as the *B. lepra* and the smegma. Stitt,[8] however, states that he has found smegma bacilli often to be quite resistant to alcohol.

In Experiment 6 it was found that the tubercle bacillus requires no iodine to enable it to retain the original Gram stain in the face of alcohol decolorization. In Experiment 8 it was seen that the tubercle bacillus retains the Gram dye (aniline gentian violet) not only when alcohol, but also when acid alcohol is applied. Hence, it must be concluded that the tubercle bacillus does not possess the typical Gram property, in fact the Gram dye (aniline gentian violet) actually proves to be acid-fast for this organism, since it is not decolorized by 3 per cent HCl in 70 per cent alcohol. The other dyes sometimes employed for Gram staining such as crystal violet and formalin gentian violet do not even adequately stain the organism. As seen in Experiments 1 and 2 the only one of these Gram dyes that will penetrate the tubercle bacillus when applied cold is aniline gentian violet. Not even carbolfuchsin will do so. In short, the acid-fast property of the tubercle bacillus precludes the possibility of its possessing the real Gram property.

From these staining experiments one is led to believe that both the acid-fast property and the "gram-positivity" of the tubercle bacillus are attributable to the same substance or substances in this organism. In order to ascertain this, tubercle bacilli were exposed (Experiments 10 to 17) to a 25 per cent solution of sodium hydroxide and to concentrated sulphuric acid with the following results: (1) Complete removal of the acid-fast property and at the same time of the "gram-positivity." This would seem to indicate that both properties depend upon the same substance or substances. This appears to be confirmed further by Experiment 18 in which the ether extract retains both the carbolfuchsin and the aniline gentian violet of the Gram staining method. (2) When tubercle bacilli are exposed for a very short time to strong acid or alkali, they may still remain acid-fast, but when stained according to Gram, part of the individual bacillus may stain "gram-positive" in the granular portion of the organism, and part of the bacillus stains gram-negative in the areas between the granules. This merely confirms the previous finding, under (1), of the identity of the acid-fast and of the "gram-positive" material. A change in the "capsule" in the intergranular area seems to have been produced by acid and by alkali, permitting the passage of the Gram counterstain where ordinarily the Bismarck brown does not penetrate, it being the most resistant portion of the tubercle bacillus to stain. The granules on the other hand are the acid-fast areas of the organism. (3) When tubercle bacilli were treated with concentrated sulphuric acid for a short time, droplets appeared outside of the organisms which retained both the carbolfuchsin and the aniline gentian violet in the face of acid-alcohol decolorization, they were acid-fast, still more confirming the opinion that both acid-

fast and "Gram" properties depend upon the same substance when applied to the tubercle bacillus. (4) Tubercle bacilli deprived of their acid-fast material by means of alkali or acid, stain gram-negative in the true sense, that is, they are decolorized by alcohol after previous application of Gram's iodine solution.

In studying the morphology of tubercle bacilli that have been deprived of their acid-fastness a striking resemblance was observed between them and young nonacid-fast tubercle bacilli in that: (a) their morphology is that of uniform rods with rounded ends and of varying lengths, but of remarkably uniform diameter, which latter is much greater than that of the average tubercle bacillus (see Plate I); (b) uniform distribution of stain throughout the bacillary body, with entire absence of beading. The bacilli are less bizarre than is the acid-fast form of organism. One is consequently led to conclude that when the tubercle bacillus is stripped of its unusual property that sets it apart from most other pathogenic organisms, namely, its acid-fastness, it is gram-negative. When the young nonacid-fast tubercle bacillus assumes acid-fastness, one may picture it as becoming impregnated, or perhaps encrusted with a waxy lipoidal covering which does not always distribute itself uniformly but often in beaded fashion and is bound to the capsule in a physicochemical union. From the manner in which the acid-fast material is removed by sulphuric acid and by sodium hydroxide, as seen in this work, leaving a morphologically intact bacillus underneath, it would seem likely that this material merely forms an investment over or within the capsule, but not within the bacillary protoplasm, otherwise one would expect a disturbance in the morphology of the bacillus consequent upon its removal.

CONCLUSIONS

1. The only Gram dye that adequately stains the tubercle bacillus when applied cold is aniline gentian violet. This dye has greater penetrability than the classical carbolfuchsin of the acid-fast technic which has to be applied hot.

2. It has been found in the above work that when aniline gentian violet used in the classical Gram stain has been applied, the tubercle bacillus will retain this dye not only after application of alcohol but also after decolorization with acid alcohol (3 per cent HCl). Consequently this Gram dye proves to be acid-fast for the tubercle bacillus.

Thus it will be seen that the acid-fast form of the tubercle bacillus *possesses no true gram-positive property* because the acid-fast property renders this impossible since none of the other Gram dyes penetrates the organism under like circumstances (applied cold).

3. When the tubercle bacillus is completely deprived of its acid-fast property either by strong acid or alkali, it becomes gram-negative, similar to young cultures which likewise often contain nonacid-fast tubercle bacilli that stain gram-negative. On the other hand, so long as the tubercle bacillus retains the "Gram" stain, it will also invariably be found to be acid-fast, if not with carbolfuchsin, at least with carbol or aniline gentian violet.

4. When the tubercle bacillus is deprived by acid or alkali of its acid-fast property, it loses its beading and stains uniformly, becomes gram-negative, and resembles the young nonacid-fast form of the organism.

5. The suggestion is herewith offered that in the future, writers of books and articles on tuberculosis when describing the staining properties of the tubercle bacillus state that in its acid-fast pathogenic form it does not possess true Gram property; that the Gram technic, as a differential stain, cannot be applied to this organism because of the presence within it of the acid-fast material.

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REFERENCES

1. Mollers, B., in Kolle, Kraus, Uhlenhuth: Handbuch der pathogenen Mikroorganismen, 1930.
Bergey, D. H.: Manual of Determinative Bacteriology, 1930, p. 497.
2. Sweany, H. C.: Am. J. Tuberc. **17**: 66, 1928.
3. Ehrlich, Paul: Deutsche med. Wchnschr. 1882.
4. Benians, T. H. C.: J. Path. & Bact. **17**: 199, 1912-13.
5. Ruhland, G. H.: Method described in Todd and Sanford's Clinical Diagnosis and Laboratory Methods, ed. 6, p. 657.
6. Long, Esmond, R.: The Chemistry of Tuberculosis, p. 91, 1923.
7. Sherman, Hope: J. Infect. Dis. **12**: 249, 1913.
8. Stitt, E. R.: Practical Bacteriology, Blood Work and Animal Parasitology, ed. 7, p. 130, 1927.

VARIATIONS OF THE SOLUBILITY OF THE CERVICAL MUCUS IN RELATION TO THE MENSTRUAL CYCLE*

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IN A VERY painstaking series of experiments Kurzrok[1] has investigated the lytic action of semen on cervical mucus. He found that normal semen digests normal cervical mucus and suggests that either changes in the lytic action of the semen or in the character of the cervical mucus or both may produce sterility. Mucopurulent cervical secretion for instance Kurzrok has always found to be nondigestible by semen.

In the course of examining sterile couples I have repeated some of Kurzrok's experiments using mainly the gross method of immersing the cervical mucus in the semen. I consider this gross method less subject to misinterpretation than the microscopic penetration method as far as the solution or digestion of the cervical mucus is concerned.

The results of my experiments were not quite as expected and appeared at first to be rather fortuitous. Thus using a normal semen from a clinically fertile man on four different, apparently normal, mucus shreds from the cer-

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vices of four women I found varying degrees of solubility. At the same time the microscopic penetration method showed, to my eyes at least, no significant variations as far as the penetration of the cervical mucus by the spermatozoa was concerned. Again in four sterile couples where I used the husband's semen with the apparently normal cervical mucus of the wife and also on three of these the semen of the known fertile man my results were inconclusive since the semen from the normal man did not produce much dissolution of the cervical mucus from two of the four women who were apparently normal. However in three cases where the two semen specimens had been used similar results were obtained with both semen samples.

As far as infected mucus was concerned I obtained practically no digestion of it with semen by the gross method but did see penetration of the spermatozoa into the mucopus. The degree of penetration seemed to depend solely on the viscosity of the mucopus which thus constituted a mechanical barrier to the progress of the sperms. With rather fluid pus penetration of the sperms was evident and I have shown before[2] that pus itself does not inhibit the spermatozoa. That the gross digestion of the cervical mucus by the semen did not at times agree with the penetrability of the same cervical mucus by the sperms may easily be explained by assuming that the fibrin and pus in the specimen may form a network or a sort of skeleton enmeshing the mucus itself. So even if the mucus itself were dissolved no gross changes would be prominent in the original specimen from the cervix. This theory was verified to some extent by the microscopic appearance of the mucus shred after it had been subjected to the influence of the semen for some time. At any rate there was less free mucus in the cervical mucus specimen of this type after immersion in the semen for a while than there had been before.

The assumption of the penetrability of the cervical mucus by the spermatozoa varying directly as its viscosity agrees very well with clinical experiences since markedly infected cervices in which the canal itself is not plugged by the tenacious mucopus frequently allow of pregnancy whereas those cervices which are plugged up solidly by a thick mucopurulent shred prevent pregnancy just as long as the condition remains unrelieved, other conditions of course being normal.

Thus the penetration of the cervical mucus seemed to me to hinge more on the viscosity of the mucus or mucopus itself than upon the lytic action of the semen. At the same time no one will deny that, just as the number, the motility, the morphology, and the biometrics of the sperms may vary so also may the lytic action of the semen be variable. Thus while far from denying any of the interpretations Kurzrok made in his experiments I could not from my own few cases (11 at that time) see any correlation between the solubility of the cervical mucus in the semen and fertility except so far as the viscosity of the cervical mucus when marked prevented the ascension of the sperms but this was then a purely mechanical and not a chemical process.

Some time after these experiments were made I had occasion to try out again the solubility of the cervical mucus in semen in investigating another sterile couple. I found that the cervical mucus which was apparently normal

was only slightly soluble in the semen of the husband. As a control I used the same cervical mucus with the semen of a known clinically fertile man and again obtained the same result. As a second control I tested out the same two semens on the cervical mucus of the perfectly normal wife of the fertile man and obtained a fairly marked digestion of the mucus by both semen specimens. These results now could mean only one of three things, either my technic was wrong, or there existed selective fertility inasmuch as a particular normal semen would not dissolve one normal specimen of cervical mucus whereas it would attack another such normal specimen, or there were undetected factors acting on the cervical mucus or the semen which would influence the reactions obtained.

As far as my technic was concerned I could discover no flaws in it but rechecked the findings the next day by means of the microscopic penetration method. However, this method did not offer any solution of the problem. My gross method was the same as that used by Kurzrok except that I used undiluted semen since dilution not only diluted the semen but also changed the osmotic pressure, the buffer reaction of the semen, etc. However, using the semen undiluted should if anything have made my results more accurate instead of confusing.

As far as selective fertility is concerned I have discussed this question in previous papers.[3] Since all the known human races do produce fertile offspring by interbreeding, it seems illogical to assume that nature would deliberately limit procreation to certain chance combinations of a special class such as a particular type of semen combined with a particular type of cervical mucus. A similar attempt to explain some cases of apparently selective fertility has also been made in connection with the various blood groups but investigations have showed no foundation for this theory. The explanation of selective fertility is usually very much simpler (see Moench, l.c.).

There remained then only the third possibility, that of some undetected change in either the semen or the cervical mucus.

Two weeks later I was able to recheck my findings on the same patients and obtained the unexpected result that the cervical mucus of the childless woman was now more soluble in the two semen samples than before and more soluble than the mucus from the cervix of the fertile woman. This latter sample of mucus indeed was very little affected by the semen. A repetition of the tests the next day again gave the same results but a different specimen of mucus showed some solubility in the semen. This could only mean that the cervical mucus in both of these women had undergone some change. On checking up the history of the patients it was found that in the first experiment the sterile woman had been in the premenstrual period and the fertile one in the first half of the menstrual cycle whereas two weeks later the conditions were of course reversed.

In view of these results I next tried out cervical mucus from two pregnant women in the seventh and ninth month of pregnancy respectively with the semen of the same fertile man and also with the semen of another normally fertile man but obtained no dissolution of the cervical mucus whatsoever. The cervical mucus from two cases of amenorrhea was also tested but the

results were inconclusive. One specimen of mucus showed some digestion, the other none at all. However, we were not in the position to determine the amount of female sex hormone in the blood of the two patients and without this determination can draw no conclusions.

Unless then we assume that the lytic action of the semen varies without any apparent cause we must draw the conclusion that the solubility of the cervical mucus varies with different times of the menstrual cycle. This is not a difficult thing to believe. Even clinically the cervical mucus in the same woman seems at times to be more fluid than at other times. It is known that follicular fluid and also the normal secretion of the fallopian tubes and the uterine body[4] dissolve the cervical mucus. In fact the rubbery consistency of the cervical plug during pregnancy has been explained as being due to the absence of the solvent action of the combined tubouterine secretions. That these secretions, especially the uterine one, are dependent more or less on ovarian activity is well known. Thus the concept of the varying properties of the cervical mucus is nothing new. Perhaps we have in addition a direct action of the ovary through the folliculin in the blood stream on the cervical mucus.

The few cases presented thus seem to point to a variation in the properties of the cervical mucus dependent apparently on ovarian activity. At the same time proper specimens are hard to obtain, the work requires much time, and there are so many pitfalls that one must hesitate to make more than tentative assumptions. I present these few cases only because they may point the way for further investigations and may prevent too hasty and incautious interpretations of the findings obtained by this method of investigation of sterile couples.

REFERENCES

1. Kurzrok, R., and Miller, Edgar G.: Biochemical Studies of Human Semen and Its Relation to Mucus of the Cervix Uteri, Am. J. Obst. & Gynec. **15**: 56, 1928.
2. Moench, G. L.: A Consideration of Some of the Aspects of Sterility, Am. J. Obst. & Gynec. **13**: 334, 1927. Some Phases of the Problem of Sterility, Med. Herald & Phsioth. **47**: 61, 1928.
3. Moench, G. L.: A Consideration of Some of the So-called Obscure Causes of Sterility, J. A. M. A. **94**: 1204, 1930.
4. Moench, G. L.: Genital Floor in the Female, Arch. Therap. **5**: 356, 1926.

30 EAST 58 STREET.

A CLINICAL STUDY OF BLOOD IRON AND HEMOGLOBIN*

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WE ARE approaching this subject with a healthy skepticism, born of the belief that, in spite of the great progress that has been made in hematology during the past few years, we are still surrounded by a mass of doubtful and uncertain data that leaves us far from a simple and satisfactory working basis. One must respect and accept as authoritative the original work of Haden,[1] Osgood and Haskins[2] and others for their valuable contributions. We cannot but feel that our immediate clinical need is the adoption of simpler methods to remove us from much of the chaos that for the past few years has served to confuse us. We believe that for many years at least the estimation of the number of red blood cells and the hemoglobin per given quantity of blood, as well as their relationship to each other, will constitute the chief desideratum in hematologic studies. One must also give greater thought to the question of iron in its relationship to hemoglobin.

It seems unnecessary to further stress the great importance of reporting the amount of hemoglobin in grams per 100 c.c. of blood, but a citation of a few of the existing errors will not be amiss. For instance one of the Sahli instruments is so graduated to indicate 100 per cent hemoglobin as containing 17.2 gm., one of the Dare instruments to contain 13.8 gm. of hemoglobin, representing a difference of more than 20 per cent, perhaps better shown by the equation, viz.:

17.2 gm. Sahli = 100 per cent
17.2 gm. Dare = 124 per cent

It should be known also that there is a wide difference in various Sahli instruments as well as in the Dare instruments. Certainly percentage reports are meaningless and even when accompanied by the name of the method used are not accurate unless such instrument has been standardized with one of the more accurate methods to be mentioned. What should be accepted as a normal average figure for the number of grams of hemoglobin contained in 100 c.c. of blood, and what should be accepted as a normal average figure for the number of red blood cells per cubic millimeter of blood? Personally, we believe that no single figure will serve the purpose with even a fair degree of accuracy. Such figures are ordinarily obtained by taking an average for a large number of so-called normals. In this number we will find a range of from 11.0 to 16.5 (a difference of 34 per cent) gm. of hemoglobin per 100 c.c. of blood, and a range of from 4,000,000 to 6,000,000 (a difference of 34 per cent) for the number of red blood cells per cubic millimeter of blood. Certainly such varia-

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tions are too great to permit of accurate averaging. The question naturally arises as to what is normal blood. An individual may be subjectively healthy and with perfectly normal functional tests of all kinds and still have comparatively low hemoglobin and red cell counts. Such findings are not uncommon among nurses, technicians, secretaries, etc., and such individuals constitute a large percentage of our so-called normal series. Conversely an individual may have advanced heart, kidney, lung, gastrointestinal, or other pathologic conditions with blood examinations much nearer the generally accepted average than the class previously mentioned (Table IV). These individuals are not used in the normal series.

Both the quantity of hemoglobin and the number of red blood cells are variables, depending probably upon age, sex, climate, food, exercise, and barometric pressure. One of us (J. D. McN.) examined the blood of one patient daily for a month and hourly for a day and found the red blood count to vary from 5,128,000 to 6,176,000 and the hemoglobin from 11.3 gm. to 15.3 gm. With such variations why not abolish such terms as "normal averages" and substitute "a normal range" for hemoglobin and red blood cells as we do for the chlorides, sugar, cholesterol, and the nitrogenous products of the blood.

This study includes about one hundred examinations of the blood made upon 81 individuals (Table I). We are unable to explain the difference between the average number of red cells in the normal bloods of our series and the normal bloods reported by others. The fact remains, however, that a count of five million is a rare exception rather than the rule, with a large percentage running from four to four and a half million. Each of these counts, as well as

TABLE I

BLOOD STUDIES IN CASES WITH A VARIETY OF CONDITIONS

CASE	SEX	AGE	DIFFERENT LABORATORIES		BLOOD IRON	HEMOGLOBIN DETERMINATIONS			COLOR INDEX HB.	IRON INDEX	DIAGNOSIS
			R.B.C.	R.B.C.		FROM IRON	O. & H.	N.	R.B.C. × 3		
1	F	36	4,096	4,340	43.2	12.7	12.0	13.0	0.93	10.5	Normal
2	M	41	4,312	4,670	46.5	13.6	13.6	13.9	0.93	10.3	Normal
3	F	45	4,200		41.5	12.1	12.1		0.87	9.8	Syphilis
4	M	69	4,430	4,310	48.5	14.3	14.3	13.2	0.98	10.9	Cholecystitis
5	F	53	4,232		42.6	12.5	12.5		0.87	10.0	Hypertension
6	F	24	4,240	4,510	39.8	11.6	11.5	11.8	0.82	9.4	Normal
7	M	64	4,536		47.5	13.9	14.0		0.91	10.4	Normal
8	M	54	4,720	4,355	48.4	14.2	14.2	12.9	0.90	10.2	Normal
9	M	45	4,312	4,445	43.0	12.6	12.3	13.1	0.87	10.0	Neurosis
10	M	57	4,304	4,321	43.0	12.6	12.8	13.3	0.87	10.0	Hypertension
11	F	27	4,496	4,300	45.4	13.4	13.3	12.8	0.90	10.1	Hydronephrosis
12	F	34	4,480	4,470	41.6	12.1	12.0	13.3	0.81	9.0	Neurosis
13	F	67	4,680	4,700	47.0	13.8	14.0	13.6	0.88	10.0	Normal
14	F	65	4,408	4,360	42.8	12.5	12.8	13.3	0.84	10.6	Hypertension
15	F	35	4,256	4,320	42.5	12.4	12.4	12.2	0.87	10.0	Surg. menopause
16	M	58	4,208	4,120	43.5	12.8	13.1	12.4	0.90	10.3	Hypertension
17	F	20	4,576	4,520	46.8	13.7	14.0	13.2	0.89	10.4	Normal
18	F	22	4,288	4,350	41.3	12.1	12.3	13.0	0.88	9.8	Normal
19	F	53	4,096	4,363	42.9	12.6	12.8	12.9	0.90	10.2	Normal
20	F	55	4,208	4,376	40.8	11.9	12.1	13.0	0.84	9.5	Arthritis
21	F	47	4,480	4,350	48.0	14.1	14.0	13.3	0.93	10.9	Migraine
22	F	27	4.384	4,380	44.5	13.1	13.4	13.2	0.88	10.3	Normal

hemoglobin determinations, have been made independently by two different workers in different laboratories, using standardized equipment, and discarding results when the two counts did not come within the average limits of error. Furthermore, the work was done by skilled hematologists and not by the usual laboratory technicians. The experience of other reliable laboratories convinces us that in Kentucky at least a normal red cell count of five million is the ex-

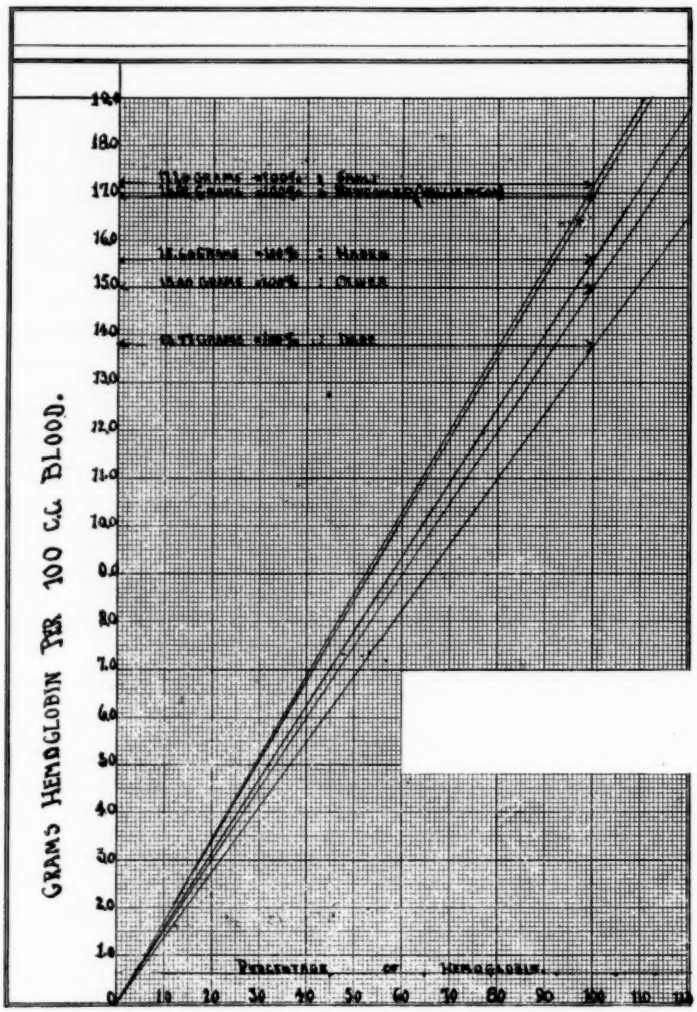

Chart 1.—Comparing hemoglobin from iron (Kennedy) with Osgood and Haskin's method.

ception and the average is from 4.3 to 4.5 million, depending largely upon the source of the normals.

Greater accuracy attends the proper determination of the hemoglobin when the method used has been carefully checked with the accurate oxygen capacity method of Van Slyke,[3] or the iron method of Kennedy or Wong. Inaccuracy of various methods has been stressed by Osgood and Haskins,[2] Karshan and Freeman,[4] Haden,[1] Lindsay Rice and Sellinger,[5] Robscheit,[6] and

others. The chief objection to the acid hematin methods seemed to lie in the time required for development of the color, fading of standards, variation in standard discs, and difficulty in reading small quantities of hemoglobin. The Osgood and Haskins is probably the most accurate of the acid hematin methods provided the standard solution is properly prepared and the proper corrections made for variations in temperature. The Palmer[7] method is quite accurate if the standard is frequently checked. The photoelectrometer of Sanford and Sheard[8] and the spectrophotometer of Haden[9] are apparently accurate methods, checking closely with the oxygen capacity method or the iron method. Although quantitative determinations of iron in the blood were made as early as 1820[10] and at long intervals following that, the most recent by Berman in 1918,[11] Brown in 1922,[12] Wong in 1928,[14] and Kennedy in 1927,[15] the method received little attention until its clinical application by Powers and Murphy.[16]

Of the methods of Wong[14] and Kennedy[15] the latter points out that Wong, like others, has overlooked the question of dissociation and continued to work with aqueous solutions. The results, however, by the two methods check so very closely that the criticism may well be ignored.

Using Kennedy's[15] method Powers and Murphy[16] found the amount of iron in the blood to be within singularly narrow and standard limits for normal adults. They consider it the most accurate criterion yet devised for evaluating the true status of patients with secondary anemia. In a later article Murphy, Lynch and Howard[17] reported a large number of blood iron determinations in normal individuals, and also in the primary and secondary anemias, as well as a variety of other conditions. For normal young men they found an average of 44.84 and for young women an average of 42.84 mg. per 100 c.c. of blood. Using the same method, we find very close agreement with an average of 44 for all normal cases. As would be expected, the results parallel closely the hemoglobin curve in all cases. They also suggested the term "iron index" (obtained by dividing the mg. of iron in 100 c.c. of blood, by the number of red blood cells in millions per 1 c.mm. of blood) to represent the amount of iron in the individual red cell. They found for this an average of 8.4 (strictly speaking 8.4 mg. $\times 10^{-11}$) for normals, and following in a general way the color index curve. Since our red blood counts are lower on an average, the average normal index is higher, being as consistently 10 in our series as the 8 4 of their series (Tables I and IV).

Both Kennedy[15] and Wong[14] have used the iron content of the blood for accurately determining the amount of hemoglobin. When it is possible to obtain results for the iron content of tissue or fluids, that are accurate to the third decimal and with the molecular weight of the hemoglobin molecule known (though the structure may be in doubt), it becomes immediately apparent that the quantity of iron expressed in milligrams divided by the iron portion of the hemoglobin molecule 0.334 by Wong and 0.334 by Kennedy) can scarcely be exceeded for accuracy when properly decimated to represent the quantity of hemoglobin in gm. per 100 c.c. of blood. The work of Riecker,[18] however, seems to prove that in normal individuals there is an average of 1.1 mg. (± 0.022) of

iron in the blood serum, and this quantity is increased in pernicious anemia particularly and slightly decreased in certain other conditions. In our study we have assumed (possibly wrongly) that cognizance of this nonhemoglobin iron

TABLE II

PRIMARY DISEASES OF THE BLOOD

CASE	SEX	AGE	DIFFERENT LABORATORIES R.B.C.	DIFFERENT LABORATORIES R.B.C.	BLOOD IRON	IRON INDEX	COLOR INDEX HB. R.B.C. × 3	HEMOGLOBIN DETERMINATIONS N.*	HEMOGLOBIN DETERMINATIONS O. & H.*	HEMOGLOBIN DETERMINATIONS FROM IRON	DIAGNOSIS
29	M	57	2,896	2,970	30.0	10.9	0.90	8.3	8.3	8.7	Acute myelogenous leucemia (autopsy)
33	F	61	3,592	3,400	30.8	8.6	0.75	9.2	8.7	8.9	Banti's disease
33	F	61	3,880	4,260	34.5	8.9	0.78	13.0	9.9	10.0	After splenectomy
35	M	8	2,240		22.2	9.9	0.85		6.1	6.4	Sickle cell anemia
								13.6	14.4	13.4	Pernicious anemia (remission)
44	F	59	4,328	4,450	45.5	10.6	0.91				
45	F	71	4,032	4,100	42.3	10.6	0.91	12.8 Sahli	12.6	12.4	Pernicious anemia (remission)
67	M	75	992	1,020	14.3	14.4	1.20	5.5	4.0	4.0	Pernicious anemia
70	M	60	1,792	1,760	21.9	12.2	1.05	4.8	6.4	6.3	Pernicious anemia
71	M	65	3.520	3,000	39.0	11.0	0.97	7.07	11.6	11.4	Pernicious anemia
72	M	55	1,994	1,960	29.5	14.3	1.29	5.90	8 8	8.6	Pernicious anemia
76	F		1,104		18.4	16.6	1.41		5.5	5.2	Pernicious anemia
73	F	18	2,688	2,850	23.3	8.7	0.74	5.5	6.9	6.7	Chlorosis
37	F	51	3,280	3,490	32.0	8.3	0.72	9.0	9.0	9.3	Chronic lymphatic leucemia, lobar pneumonia
			7,504	7,420	75.5	10.0	0.88	21.0	21.8	22.3	
			7,840	7,620	72.5	9.3	0.81	21.0	21.0	21.4	Polycythemia vera
			7,968	7,820	80.6	10.2	0.90	22.0	22.8	23.9	Phenylhydrazine
77	F	56	7,264		70.0	9.7	0.85		20.2	20.7	gr. IV daily
			7,200		65.0	9.0	0.80		19.6	19.2	Determinations
			6,984		60.6	8.8	0.78		18.2	17.9	weekly intervals
			3,872	3,872	37.8	9.7	0.87	13.5	11.3	11.0	

*N = Newcomer, O & H. = Osgood & Haskins.

Red blood cells expressed in millions per 1 c.c.: Blood iron in milligrams per 100 c.c.: Hemoglobin in gm. per 100 c.c.: Iron Index = Fe. (gm.) ÷ R.B.C. (millions): Color Index = Hb. (gm.) ÷ R.B.C. (millions) ×3: Hemoglobin from iron calculated viz.: Mg. blood iron per 100 c.c. – mg. serum iron ×3.

TABLE III

DISEASES WITH ABNORMAL BLOOD

CASE	SEX	AGE	DIFFERENT LABORATORIES R.B.C.	DIFFERENT LABORATORIES R.B.C.	BLOOD IRON	IRON INDEX	COLOR INDEX HB. R.B.C. × 3	HEMOGLOBIN DETERMINATIONS N.*	HEMOGLOBIN DETERMINATIONS O. & H.*	HEMOGLOBIN DETERMINATIONS FROM IRON	DIAGNOSIS
24	F	43	3 904		35.4	9.0	0.78		10.3	10.3	Nutritional edema
31	F	15	2,728	2,770	27.7	10.0	0.87	7.5	8.0	8.0	Hemorrhagic nephritis (autopsy)
34	F	60	3,784	3,784	37.5	9.9	0.87	11.6	11.1	11.0	Duodenal ulcer; pyloric obstruc.
38	F	2 wk.	2,984		39.5	13.7	1.16		11.0	11.5	Hydrocephalus; secondary anemia
40	M	40	3,520	3,520	35.1	10.0	0.87	12.0	10.0	10.2	Cerebrospinal syphilis —secondary stage
74	M	70	4,640	4,410	46.1	10.0	0.87	14.0	13.9	13.5	Nephritis; pneumonia
50	F	24	3,840	3,810	37.4	9.7	0.93	12.4	10.4	10.9	Colitis; focal infection
58	F	37	3,856	4.300	40.0	10.5	0 90	12.8	11.7	11 7	Colitis; neurosis
68	F	53	3.896	3.910	38.8	10.0	0.87	12.0	11.6	11 3	Secondary anemia
80	M	47	3,520	3,525	37.0	10.5	0.91	11.5	11.0	10.8	Duodenal ulcer: bleeding

*N = Newcomer. O. & H. = Osgood & Haskins.

Footnote same as in Tables II and IV.

TABLE IV

DISEASES WITH NORMAL BLOOD

CASE	SEX	AGE	DIFFERENT LABORATORIES R.B.C.	DIFFERENT LABORATORIES R.B.C.	BLOOD IRON	IRON INDEX	COLOR INDEX HB. R.B.C. × 3	HEMOGLOBIN DETERMINATIONS N.*	HEMOGLOBIN DETERMINATIONS O. & H.*	HEMOGLOBIN DETERMINATIONS FROM IRON	DIAGNOSIS
23	M	47	4,800		46.7	9.7	0.85		13.8	13.7	Renal calculus Bronchiectasis
25	F	60		4,470	55.0	12.3	1.11	13.1		16.5	Adenocarcinoma ovary
32	F	55	4,112	4,270	43.0	10.4	0.91	12.2	12.2	12.6	Arthritis Focal infection
36	M	12	4,048		39.9	9.8	0.86		11.9	11.7	Typhoid fever
39	F	55	4,400		42.8	9.7	0.85		12.0	12.5	Myocarditis, mitral insuff., focal infect.
41	M	52	5,696	4,940	55.5	9.7	0.85	14.9	15.9	16.3	Hypertension Gastroenterostomy
42	F	27	4,128	4,332	41.4	10.0	0.87	12.9	12.0	12.0	Thromboangiitis oblitrans
43	F	28	4,280	4,200	40.0	9.5	0.81	12.7	12.2	11.7	Trichiniasis
46	M	68	4,304	4,480	41.5	9.6	0.84	13.1	11.3	12.1	Cirrhosis of liver Myocarditis
47	F	65	4,800		49.0	10.2	0.90		14.5	14.4	Endarteritis obliterans, gangrene
48	F	43	4,160		41.6	10.0	0.87		12.2	12.2	Cholecystitis Mild hypothyroidism
51	F	60	4,224	4,280	42.2	10.0	0.87	12.8	12.0	12.3	Acoustic tumor
52	F	29	4,280	4,320	40.4	9.4	0.82	12.9	12.0	11.8	Pelvic pathology
53	M	48	5,120	5,500	53.0	10.3	0.90	13.5	15.4	15.6	Diverticulitis colitis
54	F	51	4,608	4,330	46.5	10.0	0.87	13.5	13.6	13.6	Recurrent hyperthyroidism
55	M	50	4,488		45.1	10.5	0.88	14.6	13.2	13.2	Duodenal ulcer
57	M	58	4,728	5,510	48.8	10.4	0.90	15.3	14.4	14.3	Duodenal ulcer
59	M	20	5,056	4,480	51.0	10.2	0.88	14.1	15.3	15.0	Chronic nephritis
60	F	75	4,200		42.8	10.0	0.90		12.8	12.5	Arthritis
62	F	32	4,480	4,340	45.5	10.1	0.90	12.9	13.6	13.4	Arthritis
63	F	58	4,392	4,370	43.0	10.0	0.85	13.0	13.0	12.6	Auricular fibrillation
64	M	77	4,336	4,321	41.0	9.5	0.81	13.0	12.0	12.0	Auricular fibrillation, diabetes,
65	M	34	4,640	4,485	46.6	10.0	0.87	13.3	14.0	13.7	Duodenal stasis neurosis
66	F	61	4,480	4,350	45.4	10.0	0.88	12.8	13.4	13.3	Uterine fibroid Cholecystitis
69	F	53	4,592	4,430	45.0	10.0	0.87	13.2	13.3	13.2	Arterial hypertension
74	M	70	4,640	4,410	46.1	10.0	0.87	14.0	13.9	13.5	Nephritis, pneumonia (death in 36 hr.)
75	F	29	4,384	4,500	43.8	10.0	0.90	13.2	13.2	12.8	Cerebral lesion
Average			4,514	4,515	45.2	10.0	0.877	13.38	13.19	13.28	

*N = Newcomer. O & H. = Osgood & Haskins.
Footnote same as in Tables II and IV.

has been ignored in the methods of Kennedy and Wong, and we have, therefore, subtracted 1 mg. from the total iron content of the blood and then multiplied the result by 0.3 (equal to dividing by 0.333 –⅓, instead of the 0.334 and 0.335 used by Kennedy and Wong respectively). While this method makes a difference of less than 2 per cent it has been used with the belief that accuracy was increased to this extent. The oxygen capacity method of Van Slyke[3] compares so very closely with both the Wong[14] and Kennedy[15] methods that little or no doubt should be entertained as to the accuracy of any of the three methods. Contrary to the general belief we consider both iron methods quite simple and easily completed in about fifteen minutes. We prefer, however, the

Kennedy method[15] because it allows time for other work while the iron mixture is being digested, and, furthermore, the colorimetric readings are definitely sharper. Any of the three methods may be safely used for standardizing hemoglobinometers and checking the accuracy of other methods.

In our experience, as well as in the experience of others, the Osgood and Haskins[2] acid hematin method gives results closely paralleling the results of

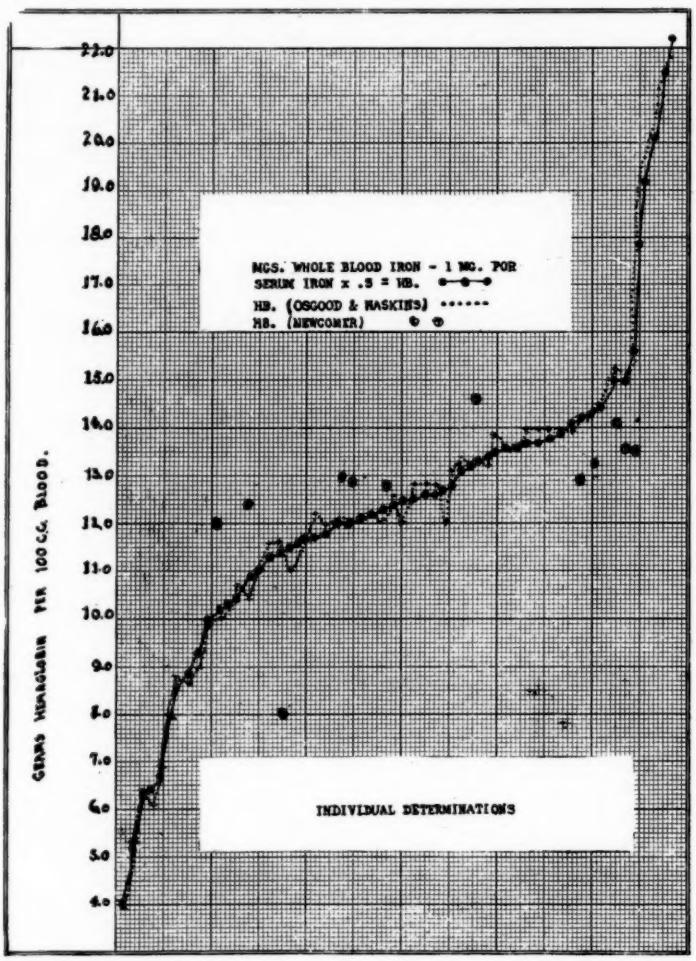

Chart 2.—Showing variation in grams of hemoglobin per 100 c.c. of blood for 100 per cent readings. May also be used for quickly converting percentage into grams.

any of the three standard methods. We believe, therefore, that a determination of the iron content of the blood, the iron content of the individual cell, and the determination of the hemoglobin by the iron method are all most valuable additions to our knowledge of hematology. They assume no averages and deal directly with the number of red blood cells and the hemoglobin found in the individual specimen. Of similar importance is the determination of the amount of hemoglobin in the individual cell. This is done by dividing the hemoglobin in grams per 100 c.c. of blood by the red cells in millions per c.mm.

of blood. The result is expressed in grams times 10^{-12}, or in billionths of a milligram per red cell, according to the following formula:

$$\frac{\text{Hb. in gm.} \times 10}{\text{red cells in millions}}$$

Example: R. B. C. 3,500,000, Hb. 10.5 gm.

$$\frac{10.5 \times 10}{3.5} = \frac{105}{3.5} = 30 \text{ billionths of a mg. per R. B. C. or } 30 \times 10^{-9} \text{ mg.}$$

The color index, determined in our series by the method of Cullen,[19] cannot be quite so accurate, since it assumes five million red cells to be normal,

TABLE V

A ROUTINE REPORT OF A BLOOD EXAMINATION (MARTIN & MCNEILL LABORATORIES)

BLOOD EXAMINATION

R.B.C.	2,896,000-2,970,000 (repeated)
Hemoglobin (Osgood & Haskins)	9.0 gm. per 100 c.c. blood (normal about 15)*
Average amount per red cell	31 billionths of a mg. (normal about 30)*
Blood iron	30 mg. per 100 c.c. blood (normal about 45)
Iron index	10.3
W.B.C.	20,050-21,050 (repeated)

DIFFERENTIAL

	Romanowsky Stain *Per cent*	*Supravital Stain* *Per cent*	*No. per c.mm.*
Myeloblasts	45.0	69.5	13,900
Promyelocytes	1.0	2.0	400
Neutrophilic myelocytes	1.0	4.5	900
Eosinophilic myelocytes	0.0	0.0	0
Basophilic myelocytes	0.0	0.5	100
Metamyelocytes	1.0	1.5	300
P. M. neutrophiles	9.5	7.5	1,500
P. M. eosinophiles	0.0	0.0	0
P. M. basophiles	0.0	0.5	100
Monocytes	1.5	6.0	1,200
Lymphocytes	2.0	7.0	1,400
Immature lymphocytes	0.0	1.0	200
Smudges	39.0	0.0	0
	100.0	100.0	20,000

Platelet count	25,000
Arneth's index	89 (normal about 60)
Reticulocytes	10%
Malarial parasites	None

The number of red cells is considerably reduced, but the average amount of hemoglobin per red cell is normal, due to an increase in the average size of the cells. Many of the large cells are polychromatic, and a number of normoblasts and megaloblasts are present. There is very little distortion.

Thirty-nine per cent of the white cells are so fragile as to be destroyed in pulling a smear. These fragile cells are identified in the supravital preparation as myeloblasts. These cells have a large round nucleus with several nucleoli and a basophilic cytoplasm. All gradations between these nongranular cells and the typical adult neutrophile can be found.

Impression: Myeloblastic leucemia.

*The percentage of hemoglobin of this patient varies according to the normal standard of hemoglobin selected—for instance, with the Sahli standard it is 52% (color index 0.9), and with the Dare standard it is 66% (color index 1.2).

and this factor enters into the equation. The results, however, conform closely with our general knowledge of what this should be, namely, high in pernicious anemia and low in secondary anemia.

It is our belief that a properly reported blood examination should contain the following data: (1) sufficient counts of the red blood cells and the white blood cells until they check within the accepted limits of error. (2) Hemoglobin determinations by the iron method of Wong[14] or Kennedy,[15] or the acid hematin method of Osgood and Haskins,[2] (3) the average amount of hemoglobin per red cell, (4) the blood iron in milligrams per 100 c.c. of blood, and (5) the iron index, (6) a supravital stain of the white cells as well as examination of the stained smear (this we believe should never be omitted and furnishes information not obtainable from the stained smear), (7) a platelet count, (8) reticulocyte count, and (9) interpretation of the findings and explanation of various hemoglobin standards (Table V). Thus it will be seen that a complete hematologic study is far from the simple procedure that has been used for years and impossible of accomplishment in the ten minutes that has been allotted and boasted for it. Most technical procedures in the laboratory have been greatly simplified without affecting their accuracy, but with our increasing knowledge of the blood, the procedure, if accurate, is becoming more difficult and more time-consuming, which is regrettable from many standpoints, the least of which is by no means financial. Because of the time consumed and the skill required it should not be included along with a urinalysis in a so-called routine examination, but accredited the same dignity that is accorded other special procedures.

REFERENCES

1. Haden, R. I.: The Normal Hemoglobin Standard, J. A. M. A. **79**: 1496, 1922. Accurate Criteria for Differentiating Anemias, Arch. Int. Med. **31**: 766, 1923.
   The Technic of Determination of the Relative Mass, the Individual Cell Volume and the Volume Index of the Erythrocytes of Man, J. LAB. & CLIN. MED. **15**: 736, 1930.
2. Osgood, E. E., and Haskins, H. D.: A New Permanent Standard for the Estimation of Hemoglobin by the Acid Hematin Method, J. Biol. Chem. **57**: 107, 1923.
   Osgood, E. E.: Hemoglobin, Color Index, Saturation Index and Volume Index Standards, Arch. Int. Med. **37**: 685.
   Hoskins, E. E., and Haskins, H. D.: The Relation Between Cell Count, Cell Volume, and Hemoglobin Content of Venous Blood of Normal Young Women, Arch. Int. Med. **39**: 643.
3. Van Slyke and Neill: J. Biol. Chem. **61**: 523, 1924.
   Newcomer, H. S.: J. Biol. Chem. **37**: 465, 1919.
   Brown, G. E., and Rowntree, L. G.: The Volume and Composition of the Blood and the Changes Incident to Diuresis in Cases of Edema, Arch. Int. Med. **35**: 129, 1925.
   Kern, R. A.: Problems of Clinical Hemoglobin Estimation, Med. Clin. N. Am. **8**: 821, 1924.
   Richard, R. J., Pierce, L. F., and Worthington, R. L.: On Hemoglobinometry, J. LAB. & CLIN. MED. **15**: 746, 1930.
   Cohen, B., and Smith, A. H.: J. Biol. Chem. **39**: 489, 1919.
4. Karshan, M., and Freeman, R. E., Jr.: A Study of Hemoglobin Methods, J. LAB. & CLIN. MED. **15**: 74, 1929.
5. Lindsay, Rice and Sellinger: A Plea for a Standardized Method of Estimating and Reporting Hemoglobin Values, J. LAB. & CLIN. MED. **11**: 737, 1925-26.
6. Robscheit, Frieda S.: A Comparative Study of Hemoglobin Determinations by Various Methods, J. Biol. Chem. **41**: 2, p. 209.
7. Palmer, W. W.: J. Biol. Chem. **33**: 119, 1918.
8. Sheard, C., and Sanford, A. H.: Photo-electrometer with One Stage of Amplification as Applied to Determination of Hemoglobin, J. A. M. A. **93**: 1951, 1929.

Sanford, A. H., and Sheard, C. S.: The Determination of Hemoglobin with the Photoelectrometer, J. Lab. & Clin. Med. **15**: 483, 1930.
9. Haden, R. L.: A New Hemoglobinometer, J. Lab. & Clin. Med. **16**: 68, 1930.
10. Le Canu, Prevost, and Dumas; quoted by Murphy, Lynch and Howard: Arch. Int. Med. **47**: 885, 1931.
11. Berman, L.: A Rapid Method for the Determination of Iron in Small Quantities of Blood, J. Biol. Chem. **35**: 231, 1918.
12. Brown, A. L.: A New Quantitative Method for the Determination of Iron in the Blood, J. Am. Chem. Soc. **44**: 432, 1922.
13. Riecker, Herman H.: Serum Iron Determinations Applied to the Study of Experimental Anemias, Am. J. Physiol. **92**: 196, 1930.
14. Wong, S. Y.: J. Biol. Chem. **55**: 421, 1923; **77**: 409, 1928.
15. Kennedy, R. P.: The Quantitative Determination of Iron in the Tissues, J. Biol. Chem. **74**: 385, 1927.
16. Powers, J. H., and Murphy, W. P.: Treatment of Secondary Anemia, J. A. M. A. **96**: 504, 1931.
17. Murphy, W. P., Lynch, Ralph, and Howard, Isabel N.: The Value of Determinations of the Iron Content of Whole Blood, Arch. Int. Med. **47**: 883, 1931.
18. Riecker, Herman H.: Iron Metabolism in Pernicious Anemia and in Secondary Anemia, Arch Int. Med. **46**: 458, 1930.
19. Cullen, G. E.: Graphic Representation of Hemoglobin and Red Blood Cell Count Ratios, J. A. M. A. **98**: 1067, 1932.

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BLOOD STUDIES IN HYPERTHYROIDISM*

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GENERAL attention was first drawn to the importance of the blood picture in hyperthyroidism by the exhaustive studies of T. Kocher[1] and his coworkers. References to the blood changes occurring in hyperthyroidism were, however, made before Kocher's studies by Ciuffini[2] and particularly by L. Caro.[3] Kocher considered the blood picture as quite typical, namely a leucopenia, neutropenia, and a lymphocytosis. These observations were confirmed by Crotti,[4] Müller,[5] Lampe,[6] Hertz and Lerman,[7] and others. Some observers, however, could not quite confirm the so-called "Kocher blood picture," e.g., Plumer in his extensive study of 578 cases. The leucopenia particularly has not been found regularly. If one considers, however, the fact that patients suffering from hyperthyroidism are frequently subject to profuse perspiration and diarrhea, which consequently leads to a certain degree of dehydration and blood concentration, one can easily understand the occasional absence of the leucopenia; in other words the blood concentration may mask the presence of the leucopenia.

The following morphologic blood studies were undertaken originally upon the request of Professor E. W. Archibald, who had made, about ten years ago, the observation, in several cases, that the blood from the thyroid vein contained a much higher percentage of lymphocytes than the blood from the thyroid artery, or the peripheral capillary blood. The difference at that time was calculated in percentages, and it was found in two cases that a percentage of between 30 and 40 in the thyroid artery, and in the peripheral capillary blood was in-

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creased to 75 and even 80 in the blood of the thyroid vein. Samples of blood were taken at the beginning of the operation, immediately upon exposure of the thyroid gland. Professor Archibald desired confirmation of the findings in these few cases. The observation seemed to indicate that a large number of lymphocytes was added to the circulating blood by the fact of its passage through the thyroid gland. With regard to this point the blood of the thyroid artery was compared morphologically with the blood of the thyroid vein in 7 cases; the blood samples were obtained during the operation.

The scope of the investigation was, however, enlarged by me to include an examination of the phagocytic power of the leucocytes, a detailed study of morphologic elements, and also blood examinations upon experimental animals. The phagocytic power of the leucocytes was studied in 6 cases of hyperthyroidism and compared with the normal.

Signs and symptoms of hyperthyroidism were produced experimentally in 3 cats by the injection of thyroxin, and morphologic blood studies made for a period of thirty days and compared with the blood of 3 normal cats.

RESULTS

Erythrocytes and Hemoglobin.—Anemia was not found to be a striking point of the picture, although low normal and slightly subnormal figures are the usual findings. A slight tendency to macrocytosis was usually observed, leading to a color index of 1 or occasionally slightly above (see Table I).

TABLE I

AVERAGE FINDINGS IN 14 CASES OF HYPERTHYROIDISM BEFORE AND AFTER THERAPY

Hb.	82% 11.48 gm.	80% 11.20 gm.
R.B.C.	4,070,000	4,030,000
C.I.	1.02	1.00
W.B.C.	4,660	5,738
Basophiles	13.8 0.3%	5.7 0.1%
Eosinophiles	105.8 2.3%	142.5 2.5%
Myelocytes	0 0	17.1 0.3%
Metamyelocytes	0 0	14.25 0.25%
Stab. forms	105.8 2.3%	228 4%
Normal segmented	1,012 22%	1,806.9 31.7%
Hypersegmented	749.8 16.3%	632.7 11.1%
Lymphocytes	2,042.4 44.4%	2,194.5 38.5%
Monocytes	455.4 9.9%	541.5 9.5%
Thrombocytes	213,291	241,333
Remarks	Preoperative	2 weeks postoperative

Total Number of Leucocytes.—The total number of leucocytes was mostly found to be low. Subtotal thyroidectomy usually led to an immediate moderate increase in the leucocyte count, which however returned to the original low level or a slightly higher one within about 2 weeks. The number of leucocytes was found in the differential count to be due to a reduction in granulocytes, especially the neutrophiles.

Basophiles and Eosinophiles.—Although normal in their percentage figures, they show low absolute numbers.

Neutrophiles.—The neutrophilic granulocytes were always found to be diminished, in percentage as well as in their absolute figures. An increased segmentation of the nuclei was a striking feature, leading to a shift to the right (Arneth-Shilling) with slight evidences of degeneration. Immediately after operation and coinciding with the temporary increase in the total number of leucocytes a considerable number of young forms appeared, returning, however, within about two weeks to almost the original formula, irrespective of the clinical condition or basal metabolism rate of the patient.

Lymphocytes.—Lymphocytes always showed a marked, but mostly relative lymphocytosis. There is a very slight relative reduction of the lymphocytosis after operation.

Monocytes.—A high percentage of monocytes was usually present before and after the operation, the presence of a monocytosis was recently pointed out by Hertz and Lerman[7] (see Table I).

Thrombocytes.—The thrombocytes usually showed low normal or slightly subnormal numbers. After operation there is a temporary increase in the number of thrombocytes, returning, however to the original level in about two weeks.

COMPARISON BETWEEN THYROID ARTERY AND THYROID VEIN

Six of the seven cases in which the blood of the thyroid artery and thyroid vein was compared were cases of hyperthyroidism with definite toxic evidences, while one of the seven cases was a case of nontoxic goiter (Tables II and III show findings in these cases).

There is a definite increase in the total number of leucocytes in the thyroid vein; this increase is due almost entirely to an increased number of lymphocytes, on the average an increase of 33⅓ per cent in the blood of the thyroid vein. Only the case with the nontoxic goiter shows an increase in the leucocyte count of the thyroid vein, which is so small, that one may have to consider it within the limit of error.

The morphology of the blood of the thyroid artery compared with that of the peripheral blood shows practically no difference. Considering the findings of pathologists, of a general lymphatic hyperplasia in hyperthyroidism with lymphatic foci in the thyroid gland itself, we can assume that this increase of lymphocytes in the thyroid vein must be due to an actual formation of lymphocytes in the thyroid gland.

Phagocytic Ability of the Leucocytes.—The phagocytic power of the leucocytes was studied by an adoption of Hamburger's method.[8] About 20 c.c. of oxalated blood was allowed to stand in test tubes, kept in an incubator at 37°,

TABLE II

BLOOD FROM THYROID ARTERY

NO. OF CASE	3	4	5	6	8	9	10	AVERAGE
Hb. %	86%	80%	83%	83%	78%	79%	92%	83%
Hb. Gm.	12.04	11.20	11.62	11.62	10.92	11.06	12.88	11.62
R.B.C.	4,310.000	3,900,000	4,165,000	4,100,000	3,880,000	3,920,000	4,710,000	4,140,000
C.I.	1	1.02	1.01	1.01	1.02	1.01	0.97	1.01
W.B.C.	5,600	4,300	5,300	6,100	5,100	4,720	6,120	5,320
Basophiles	0	0	0	61	0	0	0	5
	0	0	0	1%	0	0	0	0.1%
Eosinophiles	168	86	106	122	153	94	122	116.6
	3%	2%	2%	2%	3%	2%	2%	2.2%
Myelocytes	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Metamyelocytes	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Stab. forms	168	86	159	122	102	94	183	127
	3%	2%	3%	2%	2%	2%	3%	2.4%
Normal segmented	1,400	1,032	1,378	1,403	1,071	987	2,196	1,330.3
	25%	24%	26%	23%	21%	21%	36%	25.1%
Hypersegmented	1,008	774	954	1,159	1,020	846	549	906.3
	18%	18%	18%	19%	20%	18%	9%	17.1%
Lymphocytes	2,408	1,935	2,173	2,623	2,301	2,209	2,684	2,332
	43%	45%	41%	43%	45%	47%	44%	44%
Monocytes	448	387	530	610	459	470	366	466.4
	8%	9%	10%	10%	9%	10%	6%	8.8%
Thrombocytes	235,000	165,000	241,000	192,000	210,000	212,000	291,000	220,857
Remarks							Nontoxic goiter	

TABLE III

BLOOD FROM THYROID VEIN

NO. OF CASE	3	4	5	6	8	9	10	AVERAGE
Hb. %	85%	81%	81%	82%	77%	80%	90%	82%
Hb. Gm.	11.90	11.34	11.34	11.48	10.78	11.20	12.60	11.48
R.B.C.	4,210,000	4,030,000	4,045,000	4,130,000	3,850,000	3,790,000	4,630,000	4,097,000
C.I.	1.01	1.01	1.01	1	1.01	1.08	0.97	1.02
W.B.C.	7,000	6,000	6,900	7,800	6,200	5,860	6,670	6,632
Basophiles	0	0	69	78	62	0	0	26
	0	0	1%	1%	1%	0	0	0.4%
Eosinophiles	140	120	207	156	124	116	198	145.2
	2%	2%	3%	2%	2%	2%	3%	2.2%
Myelocytes	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Metamyelocytes	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
Stab. forms	140	60	138	78	62	58	264	112.2
	2%	1%	2%	1%	1%	1%	4%	1.7%
Normal segmented	1,470	1,200	1,380	1,482	1,178	1,160	2,112	1,419
	21%	20%	20%	19%	19%	20%	32%	21.5%
Hypersegmented	1,190	900	1,104	1,326	1,054	812	528	976.8
	17%	15%	16%	17%	17%	14%	8%	14.8%
Lymphocytes	3,430	3,120	3,381	3,900	3,162	3,132	3,168	3,333
	49%	52%	49%	50%	51%	54%	48%	50.5%
Monocytes	560	600	621	780	620	522	330	574.2
	8%	10%	9%	10%	10%	9%	5%	8.7%
Thrombocytes	217,000	196,000	218,000	185,000	236,000	224,000	278,000	222,000
Remarks							Nontoxic goiter	

for four hours. The supernatant serum was first carefully removed, and then the leucocytes which accumulate on top of the erythrocytes. The leucocytes were then placed in normal saline, washed repeatedly with normal saline changes, and finally a suspension in normal saline was made. Five-tenths cubic centimeters of that suspension was brought into contact with 5 c.c. of the serum from the same individual and incubated for one hour at a temperature of 37° C. After one hour, 0.5 c.c. of india ink was added to the suspension and incubated again for one hour. The tubes were carefully shaken every ten minutes to maintain an equal suspension. From the incubator the tubes were then transferred to an ice box for two hours, and then smears made and stained by Pappenheim's method (Jenner and Giemsa combination). In the smears 500 neutrophilic cells were counted and the number of cells containing carbon particles were marked.

TABLE IV

CASE	PHAGOCYTOSIS OF NEUTROPHILES INCUBATED IN THEIR OWN SERUM	NO. OF CASE	PHAGOCYTOSIS OF NEUTROPHILES INCUBATED IN THEIR OWN SERUM
Normal	35 per cent	2	10 per cent
Normal	38 per cent	3	12 per cent
Normal	34 per cent	5	14 per cent
Normal	32 per cent	8	12 per cent
Normal	39 per cent	13	12 per cent
Normal	37 per cent	15	14 per cent
Average	35.8 per cent	Average	12.3 per cent

The neutrophiles of the normal individuals showed an average of 35.8 per cent of the cells having stored carbon particles, while only 12.3 per cent of the neutrophiles of the patients with hyperthyroidism showed storage of carbon particles (see Table IV). The question arose now whether we are dealing with a faulty type of leucocytes in hyperthyroidism, or whether the reduction in phagocytosis was due to some factor in the serum of the patient. For that pur-

TABLE V

NO. OF CASE	PHAGOCYTOSIS OF NEUTROPHILES INCUBATED IN NORMAL SERUM (GROUP IV) %
2	28 per cent
3	30 per cent
5	32 per cent
8	27 per cent
13	28 per cent
15	29 per cent
Average	29 per cent

pose the corpuscles of the patients were incubated in the serum of a normal individual (Group IV, to exclude possible precipitations) and the corpuscles of the normal individual (Group IV) were incubated in the sera of the patients. The neutrophilic cells of the patients, previously showing an average of 12.3 per cent phagocytosis, after incubation with the normal serum showed an average phagocytosis of 29 per cent. The normal corpuscles, originally having a phago-

cytic ability of 35 per cent, after incubation with the sera of the patients showed only 13.5 per cent phagocytosis (see Tables V and VI).

From these figures it can be seen that the phagocytic ability of the neutrophiles in hyperthyroidism is definitely diminished, but this reduction of phagocytosis is not due to a faulty cell, but to the influence of the serum of the patient with hyperthyroidism, whether we are dealing with a deficient serum or whether there is a toxic inhibitory substance in the serum cannot be explained by the above data.

Experimental Hyperthyroidism.—Thyroxin was found to produce after 8 to 10 daily intramuscular injections of 1 mg. per kilo of body weight, definite

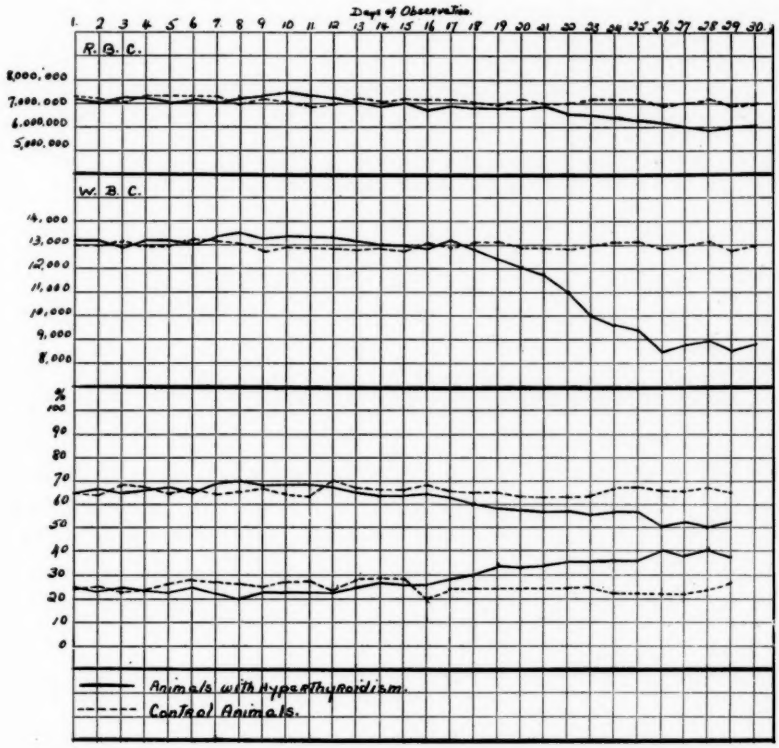

Chart I

signs of hyperthyroidism. Six cats were used for these experiments, 3 of them were given thyroxin, and 3 were used as controls. Daily blood studies were made on all animals for thirty days. The thyroxin injections were started on the seventh day and given daily until definite toxic signs developed (loss of weight, restlessness, diarrhea, etc.), then one or two injections were given weekly.

Erythrocytes.—Erythrocytes showed only slight changes. After a very slight initial rise during the third and fourth day after the injection, the erythrocytes came gradually down to a somewhat lower level during the third week after the injections had been started, leading to a slight degree of anemia. The control animals showed no changes.

TABLE VI

SERUM NO.	PHAGOCYTOSIS OF NORMAL NEUTROPHILES (GROUP IV) INCUBATED IN PATIENTS' SERUM
2	12 per cent
3	15 per cent
5	11 per cent
8	12 per cent
13	16 per cent
15	15 per cent
Average	13.5 per cent

Leucocytes.—The total number of leucocytes remained unchanged for about ten days after the injections were started, then they came gradually down to a definitely lower level. The differential count showed this reduction of leucocytes to be due to a reduction of the granulocytes. The lymphocytes showed coinciding with the reduction of the granulocytes, a mostly relative increase. The same is true of the monocytes. (Chart I shows the average percentage figures during these observations.)

RÉSUMÉ AND CONCLUSIONS

Kocher's blood picture was found to be present in practically all cases of hyperthyroidism. In addition as a striking observation an increase in the segmentation of the neutrophiles was noted, a shift to the right. Although the shift to the right may not be specific for a thyroid disturbance, just as Kocher's blood picture is not specific, it must still be considered as a considerable aid in the diagnosis, since it is not frequently observed in other pathologic conditions. The erythrocytes and thrombocytes are not strikingly diminished, but low normal or slightly subnormal figures are usually present. In other words the whole blood picture is that of a definitely depressed myeloid system. The same is apparently true in experimental hyperthyroidism. The diminished phagocytosis of the neutrophiles, being apparently due to an abnormal serum, together with the whole picture of myeloid depression all speak rather for a toxic influence of the thyroid gland. The lymphocytosis although mostly relative, is also absolute. Whether we are dealing with a general compensatory proliferation of the lymphatic tissue, or whether the proliferation is specific in the pathogenesis of the condition, cannot be decided so far. The presence of the proliferation, however, is definite and quite apparent in the comparison of the thyroid artery and the thyroid vein. I would like here to refer to the work of Hellmann, Heiber, Schlemmer, and others who believe the germinal centers of the lymphatic tissue to be a protective mechanism in the distribution of poisons and toxins. Aschoff in his excellent dissertation on the lymphatic organs[9] does not quite accept this view, since there is not enough proof for the statements; he accepts, however, the possibility of such a protective mechanism.

REFERENCES

1. Kocher, Theodor: Blutuntersuchungen bei Morbus Basedowii mit Beiträgen zur Frühdiagnose u. Theorie der Krankheit, Arch. f. Klin. Chir. 87: 131, 1908.
2. Ciuffini, P.: Ulteriori contribuo alla enotologin del morbo di Flaiain—Basedow. I and II, Policlinico sez med. 16: 284 and 364.

3. Caro, L.: Ein Fall von malignem Morbus Basedowii, Kombiniert mit den Symptomen der Pseudoleukaemie, Berlin Klin. Wchnschr. 45: 519, 1907.
4. Crotti, A.: Thyroid and Thymus, ed. 2, Philadelphia, 1922, Lea and Febiger, p. 389.
5. Müller, C.: Über morlhologische Blutveränderungen bei Strumen, Med. Klin. 6: 1340, 1910.
6. Lampe, A. E.: Die Blutveränderungen bei Morbus Basedowii im Lichte neuerer Forschung, Deutsche med. Wchnschr. 38: 1127, 1912.
7. Hertz, S., and Lerman, J.: The Blood Picture in Exophthalmic Goitre and Its Changes Resulting From Iodine and Operation, J. Clin. Invest. 11: 1179, 1932.
8. Hamburger, H. J.: Physikalisch-chemische Untersuchungen über Phagocyten, Bergman, Wiesbaden, 1912.
9. Aschoff, L.: Die lymphatischen Organe, Urban u. Schwarzenberg, Berlin, 1926.

MONILIASIS OF THE BILIARY TRACT*

Report of Case

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MONILIASIS is a disease which is caused by the Monilia fungi. There are over thirty species composing the Monilia genus.

Most of the organisms of the Monilia genus are widely distributed in nature and can be found on vegetables, fruit, wood, dead leaves, etc.

The name Monilia was first given by Hill in 1751 when he described a genus of fungi consisting of a pedicle supporting a number of naked seeds arranged together in a series like the beads of a necklace. Langenbeck in 1839 described the organism of a fungus found in the patches of Thrush in a patient who died from typhus and came to autopsy. Hansen in 1851 studied the fungi from rotting wood and found that in beer wort it produced abundant yeast cells which could produce a mycoderma skin-like infection in which the individual cells again elongated into a mycelium. But to Castellani we are indebted for his tireless investigation and classification of the members of the Monilia genus. He defines and describes this genus as follows:

"Oosporaceae, possessing in situ budding forms and mycelial threads which latter are often long and branched; in culture mostly budding forms, but sometimes filaments, in which thallospores of the blastospore type are formed. It ferments dextrose and often other carbohydrate media producing gas."

Castellani classifies the species according to color and fermentation as follows:

White, yellow, pink or red, and black.
Biochemically there are twelve groups:
1. Balcanica Group: gas produced in dextrose only.
2. Krusei Group: gas produced in dextrose and levulose.
3. Pinoyi Group: gas produced in dextrose, levulose, and maltose.
4. Metalondinensis Group: gas produced in dextrose, levulose, maltose, and galactose.
5. Tropicilis Group: gas produced in dextrose, levulose, maltose, galactose, and saccharose.

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6. Rhoi Group: gas produced in dextrose, levulose, galactose, and saccharose.
7. Bronchialis Group: gas produced in dextrose, levulose, maltose, and saccharose.
8. Guillermondi Group: gas produced in dextrose, levulose, saccharose, and raffinose.
9. Macedonionsis Group: gas produced in dextrose, levulose, galactose, saccharose, and inulin.
10. Pseudotropicalis Castellani Group: gas produced in lactose in addition to other carbohydrates.
11. Pseudolondinonsis Group: gas produced in lactose in addition to other carbohydrates, but not in lactose.
12. Zeylanica Group: no gas produced in any sugar.

The organisms vary in virulence and pathogenicity depending upon the species of the genus. In the human body they may be found on the skin surface, on the normal mucosa of the mouth and of the upper respiratory and the gastrointestinal tracts.

The Monilia genus has been shown to be the offensive organism of a number of clinicopathologic conditions of the skin, mucous membranes of the mouth, vagina, lung, and intestine.

Shelmire in 1925 summarized comprehensively cutaneous moniliasis under the heading "Thrush Infection of the Skin." Castellani gives a detail description of moniliasis involving the gastrointestinal tract, claiming that sprue is caused by Monilia. He also describes a case of purulent vaginitis caused by a Monilia genus.

It will be interesting to note that the etiology of sprue is still in a stage of medical controversy, though investigators like Ashford, Castellani, and Anderson have attempted to prove that Monilia psilosis is the offensive organism.

Moniliasis of the biliary tract has not been mentioned by the above investigators, neither has there been any article written on this subject.

However, in the course of my private practice I came across a case which I diagnosed clinically as "moniliasis of the biliary tract." The patient, female, aged forty-four years, resided in Connecticut for the past twenty-one years. While she appeared quite robust and well nourished, yet she complained of severe pain across her back, in her right shoulder and hip. She suffered with these pains for the past four years. Physical examination showed negative findings except for a slightly enlarged spleen and a tender mass in the right upper quadrant which was thought to be the gallbladder. The liver and the kidneys were not palpable. There was dryness in the mouth, but the tongue was clear. In intestinal moniliasis the tongue is coated, sore, and beefy. In this patient the bowels were constipated, and the stools were small in volume, hard and dark in color, while in intestinal moniliasis there is a pale, voluminous, foamy diarrhea. Blood Wassermann, urine, and routine blood work were negative. This patient had chills, fever, sweat on several occasions although smears for malaria, and blood cultures were negative.

The gallbladder was drained for diagnostic purposes. Before the sterile Rehfuss tube was inserted cultures were taken from the mouth, pharynx, and teeth. The result of these cultures was negative for fungi.

A sample of "B" bile was sent to the Connecticut State Laboratory for bacteriologic examination. The report showed that the bile contained *B. coli,* nonhemolytic streptococci, and yeast. On the culture the yeastlike form appeared in large amounts and was similar to Monilia morphologically. A portion of this culture was sent to the State Laboratory of Hygiene, Madison, Wisconsin. Doctor W. D. Stovall was kind enough to send a résumé of his report:

"Acid and gas in glucose, levulose, mannose. No fermentation on maltose, galactose, or saccharose. There is a distinct pellicle on the sugars which are fermented by this organism. Colony formation on malt agar was dull, dry and flat with mycelium production in forty-eight hours. This organism corresponds to the cultural relations of several strains of mycoderma which we have studied. It also corresponds to the cultural characteristics which we obtained when we studied M.° Krusei type."

The bile microscopically showed motile bacteria, bile crystals and pigments, fatty débris, epithelial cells and shreds.

The patient's gallbladder was drained for six consecutive weeks. Internally she was given heavy doses of potassium iodide and hexamethylamine. She was on a cholesterin poor diet.

It was interesting to note that the bile became free from fungi and bacteria, though it showed a few crystals and bile pigments. The patient has been relieved of backache and pain in the joints for over a year.

CONCLUSIONS

A case of moniliasis of the biliary tract is presented. The diagnosis was made by finding the fungus in the bile and on culture.

The treatment consisted of biliary drainage, potassium iodide, and hexamethylamine internally, and a cholesterin poor diet.

Patient has been symptom-free for over a year.

REFERENCES

Castellani, Aldo: Fungi and Fungous Diseases, A. M. A., C. 1927, 1928.
Jacobson, H. P.: Fungous Diseases, Thomas, 1932.

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STUDIES ON RUBBER GLOVE STERILIZATION AND THE USE OF STERILITY INDICATORS*

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FROM the beginnings of aseptic surgery the problem of adequate sterilization of the surgeon's hands has been of major importance. It soon became apparent that mechanical cleansing and chemical disinfection did not suffice, and trials were made of covering the hands with various substances. Methods,[1] such as the use of waxy materials or of cloth gloves impregnated with collodion or liquid paraffin, were devised. Rubber gloves were introduced into surgery by Halstead[2] in 1889. Their use was recommended by Robb in a textbook published in 1894, and they have been almost universally adopted since that time.

Rubber gloves are commonly sterilized by steam under pressure. This method has the advantage of being simple and efficacious, although it has not been standardized. Different practices are employed in wrapping the gloves, in stacking them in the autoclave, and in the times and pressures utilized for sterilization. The life of the gloves is relatively short, even with the best of care, as exposure to steam under pressure quickly reduces their strength and lessens their elasticity. It may easily be seen that any undue excess of steam pressure or time of sterilization will shorten the useful life of the gloves even more than is necessary.

The experiments to be reported, were planned in order to determine the minimum lengths of time that would be required, at various pressures, to completely sterilize heavily contaminated rubber gloves which had been wrapped in a uniform manner. At the same time an assay was made of the practical value of two types of sterilization indicators which are sold commercially. The indicators used were the Diack† and the Sterilometer.‡

The Diack is a small sealed glass tube containing a pellet of reddish wax-like material. When a certain temperature is reached, the enclosed pellet melts and changes color almost immediately. The temperature required for this change was found to be approximately 121° C., which corresponds to a little over 15 pounds of steam pressure. The complete melting of a Diack which has been placed near the center of a package of material to be autoclaved, is assumed to be indicative of the sterility of the material in question.

The Sterilometer is a strip of heavy paper on which has been printed a small figure representing a thermometer, the bulb and stem consisting of two sections, each of which is composed of a special cream-colored ink. On exposure to heat, these inks darken gradually. Complete blackening of the left-hand section, which is represented by the bulb and part of the stem, is said to

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‡From the Sterilometer Laboratories, 2308 Victoria Avenue, Los Angeles, Calif.

indicate the maintenance of a temperature of 230° F. for a minimum of approximately fifteen minutes, while the development of a corresponding color in the right-hand section means that 250° F. has been maintained for the same period of time. A Sterilometer, which has had both sections completely blackened by being near the center of a package of material that has been autoclaved, is assumed to indicate the complete sterility of the contents of the package in question. The blackening of the left-hand section alone does not necessarily indicate sterility. This section is merely supposed to act as a color standard and to show that the material in question has actually been autoclaved.*

The following organisms were used in the experiments: *Cl. oedematiens, Cl. welchii, Cl. septicum (Vibrion septique), Cl. sordellii, Cl. sporogenes, Cl. histolyticum, Cl. tetani, Cl. parabotulinum A, B. anthracis* and *B. subtilis.* The anaerobes were grown in chopped veal infusion broth for a period of at least two days before use. Microscopically all, save *Cl. welchii* and *Cl. tetani,* showed a prolific formation of spores. Growth was usually apparent in these latter cultures, however, even after they had been heated to 85° C. for fifteen minutes. *B. anthracis* and *B. subtilis* were grown on agar slants, and formed innumerable spores.

The gloves used were the gloves regularly employed in surgery at the Los Angeles County General Hospital. After contamination they were placed one in each side of a regulation glove book, and this was then wrapped in a double thickness muslin glove wrapper about 26 inches square. In most of the experiments four such packages were tied together before autoclaving. This made up a rather bulky bundle containing eight gloves, and sometimes a ninth glove was inserted in one of the glove books. The use of such bundles was an added factor of safety, as the penetration of steam was definitely slower than is the case in general practice, where gloves are wrapped in small packages and carefully stacked so as to facilitate the rapid penetration of steam.

The autoclave used was the instrument employed for the sterilization of bacteriologic supplies at the Los Angeles County General Hospital. This machine was carefully calibrated in respect to pressure and temperature. It was operated throughout, after a few preliminary experiments by one individual. In each experiment the glove bundles were placed as nearly as possible in the same portion of the autoclave. No preliminary vacuum was produced before the beginning of sterilization, but a special method, for quick elimination of the air, was employed in the later experiments. The door of the autoclave was left slightly open, and steam was allowed to escape at full pressure for a period of from one to two minutes. The door was then closed, and the steam pressure, used for sterilization, was attained very rapidly. This introduced an added factor of safety in that the time taken to attain a certain pressure was cut to the minimum, whereas, in general practice, this time may actually be a considerable factor in the process of sterilization.

Most of the experiments were conducted as follows: Two sterile swabs were

*The Sterilometer has recently been manufactured in a new and slightly different form. The ink in the bulb of the thermometer corresponds to that which made up the former left-hand section, while the stem consists entirely of the ink which composed the former right-hand portion. "The stem must turn black until it matches the color of the bulb," is printed in each indicator.

dipped into one of the spore-filled cultures and were then inserted into different fingers of a glove. A Sterilometer and, under certain circumstances, also a Diack were placed in the cuff of the glove. Eight, and sometimes nine, gloves, prepared in this manner, were wrapped as previously indicated and tied into one bundle. They were then autoclaved for a given time and pressure. The packages in the bundle were then opened, and the swabs were removed and cultured. The anaerobes were cultured in chopped veal infusion broth which had recently been boiled for a few minutes and then cooled. The swabs were inserted, rotated, and withdrawn, and sterile vaseline was poured over the surface of the medium. The aerobes were cultured in deep tubes of extract agar which had been melted and cooled to 40° C. Each culture was labeled with the name of the organism, the date, and the position which its swab had occupied in the bundle of gloves, i.e., whether in the outside or inside glove in an outside or inside package. One control was made directly from the original culture of each organism used. The actual Sterilometer was pasted opposite the record of its corresponding pair of cultures, and the condition of the Diack was recorded.

The cultures were incubated for at least four weeks before being discarded. Such a time is open to criticism as we realize that an occasional spore may lie dormant for an almost indefinite period before germinating. Meyer[3] recommends incubating for a three months' period when conducting spore resistance tests on *Cl. parabotulinum A*. Meleney and Chatfield,[4] however, regard fifteen days as an adequate incubation period in tests to check the sterility of surgical catgut, and we have chosen a time lying between the limits recommended above.

Growth in the anaerobic cultures was clearly evidenced by the production of gas, which forced the vaseline away from the surface of the medium. Growth in the aerobic tubes could be seen on the surface of the agar, and also, to a certain extent, in its depth.

A total of 73 experiments involving over 1,200 cultures, exclusive of controls was performed, all of the cultural work being done by the author. In the early experiments a different organism was used to contaminate each glove in the bundle. An effort was made to distribute the different organisms so that, in a series of experiments, each one would be placed near the inside and near the outside of the bundles an approximately equal number of times. It subsequently became evident, however, that *Cl. oedematiens* was by far the most heat-resistant of the organisms being used. In the experiments in which comparable numbers of the different cultures were employed, 14 out of 76 or 18 per cent of the cultures of *Cl. oedematiens* showed growth, whereas growth occurred in only 5 out of 76 or 7 per cent of the cultures of *Cl. sporogenes,* and the percentage of positive cultures of the other organisms was even less. For this reason all the gloves were contaminated with *Cl. oedematiens* in a number of tests which were performed at a later date.

EXPERIMENTAL RESULTS

In performing the experiments a given pressure was selected and a time which was thought to be inadequate was employed at first. This period was

then lengthened progressively, as growth in the cultures developed, until a time was found for each pressure at which all cultures remained sterile. These minimum sterilizing times were checked, with one exception, by at least three experiments, each involving eight or nine different cultures, and, in addition, three experiments in which *Cl. oedematiens* alone was used. This latter test was considered to be the most crucial of all. In two instances *Cl. parabotulinum A* was substituted for *Cl. oedematiens* in these latter experiments. The question arose as to whether dried spores might be more resistant to heat than are spores in the moist state. In answer to this, in at least one of each series of experiments, designed to check the minimum sterilization times at different pressures, dry gloves were contaminated with swabs on which the spores had been dried. These dried spores showed no more resistance to heat than did the corresponding moist spores under like conditions. Table I gives the results of this series of tests with respect to completeness of sterility attained at different times and pressures.

TABLE I

COMPLETENESS OF STERILIZATION ATTAINED AT DIFFERENT TIMES AND PRESSURES
(CONTROL CULTURES OMITTED)

STEAM PRESSURE IN POUNDS	TIME IN MINUTES	NUMBER OF EXPERIMENTS	STERILE CULTURES	CULTURES SHOWING GROWTH
5	20	2	32	2
5	30	9	149	3
5	40	6	96	0
8	30	6	96	0
10	10	1	11	7
10	15	1	16	2
10	20	7	120	0
10	25	7	118	0
15	10	1	15	3
15	15	3	49	5
15	20	3	48	6
15	25	4	66	0
20	10	4	63	3
20	15	7	118	0

An examination of the above table will show that the minimum sterilizing times attained for different pressures, under the experimental conditions employed, were as follows:

5 pounds---------- 40 minutes
8 pounds---------- 30 minutes
10 pounds---------- 20 minutes
15 pounds---------- 25 minutes
20 pounds---------- 15 minutes

It is perfectly evident that a discrepancy appears between the times which were found to be adequate at ten and at fifteen pounds pressure. This discrepancy illustrated the important fact that the pressure recorded in an autoclave is not always a reliable index of sterilization. In order to insure adequate steam penetration the complete removal of air from the chamber is essential. For this purpose most autoclaves are equipped with an automatic chamber-drain valve and steam trap which allow the escape of air and water of condensation, but hold back most of the steam. It is entirely possible for such an

apparatus to get out of adjustment, in which case air may be retained and the temperature in the center of the packages being autoclaved may be raised relatively little. In such an event, when surgical materials are being sterilized, disastrous accidents may occur. As a check to offset this possibility the use of dependable sterilization indicators would seem advisable.

In two experiments, in which gloves were autoclaved at 15 pounds for twenty minutes, growth occurred in several cultures. The Sterilometers, which had been placed in the cuffs of the gloves that were incompletely sterilized, showed only a partial blackening of their left-hand portion and almost no change at all in their right-hand section. This condition of the Sterilometers was evident immediately upon opening the packages, and it gave presumptive evidence that the gloves in question might not have been completely sterilized, which, indeed, turned out to be the case. Furthermore a check of all the tests shows that in no instance did a completely blackened Sterilometer correspond with a glove that proved to have been incompletely sterilized.

The value of the Sterilometer is further illustrated by the following experience, which recently occurred in our laboratory. Several batches of culture media were found to be contaminated after having been autoclaved in the usual routine manner at 15 pounds for twenty minutes. For this reason it seemed advisable to check the functioning of the sterilizer. Two bundles, each containing four glove packages, were made up in the regular manner. A Sterilometer was placed in each glove bundle, and one was also tied on the outside of each bundle. One bundle was autoclaved at 15 pounds for twenty minutes in the exact manner that had been employed for the above sterilization of media. The second bundle was then sterilized in the same manner, with the exception that special care was taken, in this instance, to completely exhaust all air from the chamber of the autoclave.

Fig. 1 is a photograph of the actual Sterilometers used in the above two experiments. On the left are shown the indicators taken from the bundle which had been autoclaved with special care regarding the complete exhaustion of air. These Sterilometers are all either completely or very nearly completely blackened. They are all very much darker than the darkest Sterilometer that was associated with a contaminated glove in any of our experiments, which goes to show that the penetration of steam was sufficient, in this case, to insure complete sterilization of this bundle of gloves. The Sterilometers in the right-hand column were taken from the bundle which had been autoclaved in the manner routinely employed for culture media. The Sterilometer from the outside of the bundle shows an almost complete degree of blackening. The indicators from within the packages, however, are variable and incompletely darkened, demonstrating that the penetration of steam had been insufficient to insure adequate sterilization in this instance. Such a record should serve as a warning that an autoclave is not functioning properly, a state of affairs which might otherwise be entirely overlooked for a considerable period of time.*

*These experiments were subsequently repeated using gloves contaminated with *Cl. oedematiens*. Several of the gloves, which were run in the improperly functioning autoclave, and which were associated with incompletely blackened Sterilometers were found to have been incompletely sterilized. Complete sterility was obtained in the case of those gloves which were associated with thoroughly blackened Sterilometers, a state of affairs which occurred only when all the air was exhausted from the autoclave.

The value of an indicator, such as the Diack, appears to be more problematical. In the experiments summarized in Table I, a melted Diack was always associated with complete sterilization of the glove in question. The Diacks employed in this study, however, did not melt at all until a temperature of approximately 121° C. was reached. For this reason they could not be used as indicators when gloves were autoclaved at low pressures. On the other hand they melted almost immediately when the requisite temperature was attained, and therefore, theoretically at least, did not necessarily give evidence concerning the time factor involved in the complete sterilization of spores at any given temperature.

It, therefore, seemed desirable to further assay the value of this indicator, and a new type of experiment was devised. Two gloves were contaminated with *Cl. oedematiens* and a Diack and a Sterilometer were tied together and

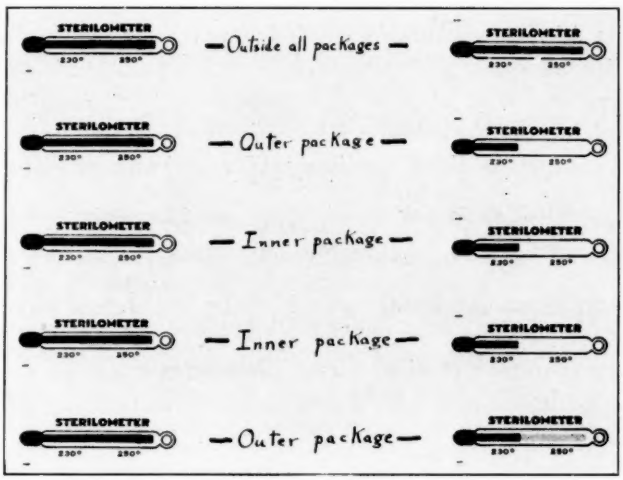

Fig. 1.

placed in the cuff of each of them. The gloves were wrapped in a small package containing only one glove book. They were autoclaved at a high pressure for a short time, following which cultures were made in the manner previously indicated. A number of these experiments were performed, the times in the autoclave and the pressures attained being varied. The protocol of one such experiment is here given:

Total time in autoclave	3½ minutes
Highest pressure attained	19 pounds
Diack	melted
Sterilometer	incompletely darkened
Culture from glove	growth

Results such as this were obtained on two occasions. In these tests the efficacy of the Sterilometer was again upheld, whereas it was shown that it is possible to demonstrate instances in which the melting of a Diack will not serve as an indicator of complete sterility.

The importance of the penetration of steam in sterilization is also illustrated by the following results: In 34 instances of cultures showing growth, a record was kept of whether the swabs, from which the cultures were made, had been taken from an inside or an outside package. Of these, 7 swabs were taken from outside packages, whereas 27 came from inside ones.

DISCUSSION

It is of interest to compare the results of the experiments reported above with previous work on the heat resistance of various spores. Bigelow and Esty[6] reported that some thermophilic organisms would withstand considerably more heat for a given time than did any of the spores employed in our work. It is obvious that the sterilization indicators used by us would probably have been inadequate as an index of sterility if the gloves had been contaminated with spores of these thermophiles. These organisms, however, do not appear to be of any consequence as a surgical problem.

The spores of *Cl. parabotulinum A* are generally considered to be more heat-resistant than are those of any other organism pathogenic to man. Meyer,[3] in 1931, quoting the work of Esty and Meyer[7] in 1922, reports the following figures as indicating the "maximum heat resistance of *Cl. parabotulinum* spores produced in a pea peptic digest medium under optimum conditions of growth:

"33 to 36 minutes at 110° C. or 6.1 pounds
11 to 12 minutes at 115° C. or 9.8 pounds
2 to 8 minutes at 120° C. or 14.1 pounds"

Although this organism can hardly be said to be of surgical significance we decided to check the Sterilometer against these maximum survival times. These indicators were therefore sterilized free in the autoclave at the longest time intervals recorded in the above table and at the corresponding pressures. None of these Sterilometers were completely blackened. It therefore appears safe to assume that a completely blackened Sterilometer would indicate complete sterilization of a glove even though it had been contaminated with an organism as resistant as the above strains of *Cl. parabotulinum A*.

Most of the organisms employed by us may be said to be of great surgical significance. Fortunately their heat resistance appears to be relatively low, when compared with that of the above bacteria. Esty and Meyer[7] reported that the heat resistance of a number of the anaerobes associated with gas gangrene was markedly less than that of *Cl. parabotulinum A*. Murray and Headlee[8] found that spores of *Cl. tetani* would be effectively destroyed in physiologic saline at the following times and temperatures:

25 to 60 minutes at 95° C.
10 to 25 minutes at 100° C.
5 to 10 minutes at 105° C.

Murray[9] found the following times and temperatures effective for the destruction of anthrax spores under like conditions:

15 to 45 minutes at 90° C.
10 to 25 minutes at 95° C.
5 to 10 minutes at 100° and 105° C.

Finally Headlee[10] reported that spores of *Cl. welchii* would be destroyed under like conditions in

35 minutes or less at 90° C.
15 minutes or less at 95° C.
10 minutes or less at 100° C.

From the work reported it seems that a completely blackened Sterilometer may, when properly used and interpreted, be regarded as an indicator of the adequate sterility of rubber gloves. This is especially the case inasmuch as this indicator has been successfully checked against more than 400 cultures of *Cl. oedematiens,* which appears to be a highly resistant organism. The penetration of steam has been shown to vary considerably when glove packages are packed closely together. For this reason it is essential that the individual glove packages be carefully arranged in the autoclave to permit the maximum access of steam to each. We feel that gloves may be safely sterilized at 10 pounds for thirty minutes, 15 pounds for twenty minutes, or 20 pounds for fifteen minutes, provided that the autoclave is functioning properly and that the penetration of steam is sufficient. A suitable indicator, if placed in the center of each glove package, should act as an adequate check as to the efficacy of the sterilization obtained under the above conditions.

SUMMARY

Rubber gloves were contaminated with different spore-forming organisms, wrapped in a uniform fashion, and autoclaved at varying times and pressures. They were then tested for sterility.

Minimum sterilizing times for gloves, autoclaved at varying pressures under the experimental conditions employed, were determined.

Cl. oedematiens was found to be the most heat-resistant of the organisms used.

The values of two indicators of sterility, the Diack and the Sterilometer, were checked in connection with these glove sterilization experiments.

The Sterilometer, when properly used and interpreted, was found to be an adequate indicator of the sterility of the gloves in question.

Gloves may safely be sterilized at 10 pounds for thirty minutes, 15 pounds for twenty minutes, or 20 pounds for fifteen minutes, provided that the autoclave is functioning properly and that adequate penetration of steam has occurred.

REFERENCES

1. Juvara, E., and Jianu, A.: Untersuchungen über die Sterilisierung der Gummihandschuhe, Wien. klin. Wchnschr. **24:** 1096, 1911.
2. Reichert, F. L.: William Stewart Halstead and His Account of the Introduction of Rubber Gloves in Surgery, Internat. Abst. Surg. **50:** 393, 1930.
3. Meyer, K. F.: The Protective Measures of the State of California Against Botulism, J. Prev. Med. **5:** 261, 1931.
4. Meleney, F. L., and Chatfield, M.: The Sterility of Catgut in Relation to Hospital Infections, With an Effective Test for the Sterility of Catgut, Surg. Gynec. Obst. **52:** 430, 1931.
5. Harper, J.: Dressing Sterilizers With Special Reference to Temperature, Pressure and Chamber Air Exhaustion During the Process of Sterilization, U. S. Naval Med. Bull. **25:** 62, 1927.

6. Bigelow, W. D., and Esty, J. R.: The Thermal Deathpoint in Relation to Time of Typical Thermophilic Organisms, J. Infect. Dis. **27**: 602, 1920.
7. Esty, J. R., and Meyer, K. F.: The Heat Resistance of the Spores of *B. botulinus* and Allied Organisms, J. Infect. Dis. **31**: 650, 1922.
8. Murray, T. J., and Headlee, M.: Thermal Death Point. I. Spores of *Clostridium tetani*, J. Infect. Dis. **48**: 436, 1931.
9. Murray, T. J.: Thermal Death Point. II. Spores of *Bacillus anthracis*, J. Infect. Dis. **48**: 457, 1931.
10. Headlee, M.: Thermal Death Point. III. Spores of *Clostridium welchii*, J. Infect. Dis. **48**: 468, 1931.

VARIABILITY IN THE CORN COMPONENT OF A RACHITOGENIC DIET

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IN A PREVIOUS communication[1] we recommend that before yellow corn (maize kernels) be used in a rachitogenic diet, it should be stored for at least six months after grinding. We have found that this procedure reduces variations in the intensity of rickets and increases the severity of the rickets produced in rats on the Steenbock diet No. 2965. Bourdillon and coworkers[2] have recently substantiated this finding.

Further work in these laboratories supports our original findings and confirms our opinion that the content of antirachitic substance in corn is responsible for the variations. This work consists of testing forty samples of yellow corn obtained during June and July, 1931, from nineteen different states of the United States. Five pounds of each sample were finely ground and stored for six months at room temperature in white, soda-glass, cork-stoppered bottles. An equal portion of each sample was stored under the same conditions but ground at the end of the storage period rather than at the beginning. After storage, a sample of each corn, stored after grinding and ground after storing, was incorporated as the sole corn component of a batch of the Steenbock ration.

These eighty rations, differing only in respect to their corn component, each prepared with the same care to secure uniformity in compounding, were fed for the usual twenty-one days to Wistar strain albino rats, of average initial weight 50 to 55 gm. in groups of four each. Every animal was weighed each second day throughout the test.

The degree of rickets was determined by the "line test" and confirmed by x-ray. The results are expressed as +, ++, +++, ++++, in increasing order of severity. The average degree of rickets for each group is displayed in Table I and in Fig. 1.

Chemical determinations on the forty corn samples included moisture, ash, fat (petroleum ether thirty-hour Soxhlet extract), calcium (modified method of Neumann[3] and of Treadwell and Hall,[4] and phosphorus (modified methods of Neumann[3] and Johnson[5]). Condensed results are given in Table I. It may well

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be that there are phosphorous linkages which vary in assimilation properties. We are analyzing for these various phosphorus fractions and will report on them later.

The most significant chemical constituents, because of the known importance of the Ca/P ratio in this diet, are calcium and phosphorus. In our analyses, the size of the sample taken (10 gm.) proved insufficient to give a quantitation of calcium. Check determinations made subsequently upon larger samples by us, and the subsequent oral presentation of corroboratory work by Holmes,[6] showing that the expectancy for calcium in corn is of the order of about 0.05 per cent, make it obvious that in this ration, in view of its large proportion of added

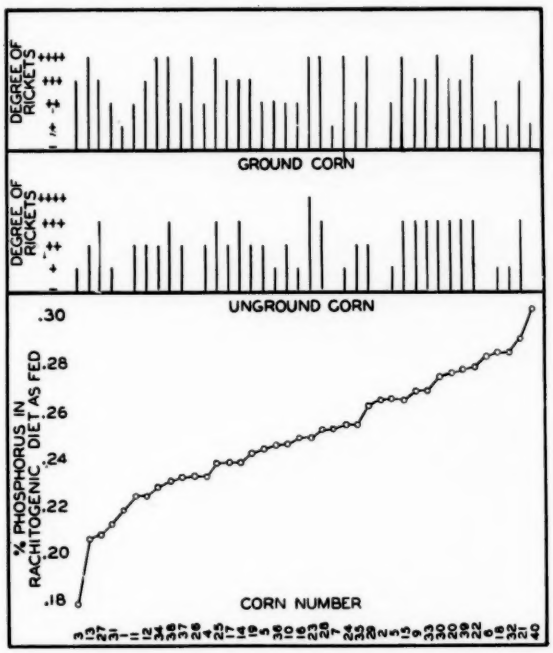

Fig. 1.

$CaCO_3$, the calcium constituent of the corn itself has an insignificant effect upon the total Ca/P ratio of the diet as compounded.

Phosphorus determinations showed that there is considerable variation in the amount of this element in the different samples of corn. This variation was so large that the Ca/P ratio of the diet compounded with sample No. 40 was 3.98/1, and 6.7/1 with sample No. 3. The degree of rickets produced by these two samples, when used freshly ground, was "none" and "one +," but when stored after grinding it was "two +" and "three +" respectively. While actual determinations of phosphorus were not made upon the same corn sample both before and after storage, there seems no valid reason for a decrease in total phosphorus (on a dry basis) during storage, and it is therefore assumed that the total phosphorous content of the stored ground corn represents the content of a similar sample stored in the whole kernel. An examination of Fig. 1 indicates that neither the total phosphorus nor the Ca/P ratio of the

TABLE I

CORN NO.	BREED AND SOURCE	MOISTURE %	DRY WT. BASIS				IN DIET AS USED: P %	AV. RAT WT. INCREASE IN 21 DAYS IN GM.		DEGREE OF RICKETS	
			ASH %	PET. ETHER SOL. SUB. %	CA %	P %		CORN UNGROUND	CORN GROUND	CORN UNGROUND	CORN GROUND
1	Haskins yellow, Mass.	8.4	1.3	4.2	–	0.312	0.218	14	10	none	slight
2	Golden glow dent, Mass.	8.2	1.6	4.5	–	0.382	0.266	28	19	none	none
3	Lancaster sure crop, N. J.	12.1	1.5	3.9	–	0.268	0.179	18	11	slight	moderate
4	Clarage, Ohio	8.4	1.4	4.6	–	0.332	0.232	12	10	fair	fair
5	Lancaster sure crop, Del.	4.0	1.4	3.7	–	0.336	0.245	14	13	fair	fair
6	Minn. 13 (Sd 86), S. Dak.	8.0	1.6	4.6	–	0.400	0.280	18	13	none	fair
7	Williams yellow dent, Mass.	8.3	1.6	4.3	–	0.364	0.253	20	8	none	fair
8	Minn. 13 Thorpe, N. D.	11.5	1.4	3.6	–	0.396	0.266	14	9	slight	fair
9	Duncan yellow dent, Mich.	11.4	1.5	4.8	–	0.400	0.269	17	19	moderate	moderate
10	Not known, Wis.	9.0	1.6	4.6	–	0.359	0.248	16	14	fair	fair
11	Thatcher's gold dent, Ark.	7.9	1.4	3.7	–	0.320	0.224	8	12	fair	fair
12	Not known, Ill.	13.7	1.2	3.9	–	0.340	0.224	9	9	fair	fair
13	Longfellow-Davis flint, Mass.	9.3	1.3	4.3	–	0.300	0.207	10	12	fair	severe
14	Not known, Ill.	12.4	1.9	4.3	–	0.358	0.238	18	16	moderate	moderate
15	Hulsart yellow dent, N. J.	13.3	1.3	4.1	–	0.404	0.266	17	14	moderate	severe
16	Kruger, Ill.	12.3	1.4	4.3	–	0.372	0.248	14	6	slight	fair
17	Golden glow, Mich.	11.6	1.5	4.1	–	0.354	0.238	15	15	fair	moderate
18	Yellow dent seed corn, Utah	12.2	1.5	4.2	–	0.427	0.284	13	13	slight	fair
19	Sure crop, Pa.	8.0	1.3	4.4	–	0.345	0.242	13	11	fair	moderate
20	Hall's gold nugget flint, N. J.	12.7	1.5	4.6	–	0.416	0.276	8	6	moderate	moderate
21	Polar dent, Mich.	11.6	1.5	4.7	–	0.432	0.290	8	6	moderate	moderate

TABLE I—CONT'D

CORN NO.	BREED AND SOURCE	MOISTURE %	DRY WT. BASIS				IN DIET AS USED: P %	AV. RAT WT. INCREASE IN 21 DAYS IN GM.		DEGREE OF RICKETS	
			ASH %	PET. ETHER SOL. SUB. %	CA %	P %		CORN UNGROUND	CORN GROUND	CORN UNGROUND	CORN GROUND
22	St. Paul 13 dent, Minn.	12.0	1.6	4.0	–	0.414	0.278	17	18	moderate	severe
23	MAC yellow dent, Mich.	11.8	1.7	4.4	–	0.370	0.248	14	14	severe	severe
24	Reid's yellow dent, Neb.	13.8	1.3	3.6	–	0.389	0.254	21	17	slight	severe
25	Golden queen, Pa.	8.2	1.5	4.4	–	0.340	0.238	15	13	moderate	severe
26	Western Plowman, Ill.	12.9	1.2	3.2	–	0.351	0.232	18	20	none	severe
27	Reid's yellow dent, Del.	13.6	1.4	4.0	–	0.318	0.208	20	13	moderate	moderate
28	Reid's yellow dent, N. J.	12.8	1.5	4.4	–	0.381	0.252	23	19	moderate	severe
29	Not known, Kan.	8.4	1.4	4.1	–	0.375	0.261	12	4	fair	severe
30	Wilson yellow dent, Fla.	12.2	1.4	4.6	–	0.404	0.273	11	12	moderate	severe
31	Somerset teaming dent, N. J.	12.9	1.2	5.0	–	0.320	0.212	9	16	slight	fair
32	Cattle, Neb.	14.0	1.5	3.8	–	0.435	0.284	16	7	fair	slight
33	King Philip Hybrid, Calif.	12.4	1.0	4.3	–	0.404	0.269	15	14	moderate	moderate
34	Pickett yellow dent, Mich.	11.7	1.5	3.8	–	0.340	0.228	17	13	fair	severe
35	Reid's white cap, N. J.	12.0	1.5	4.5	–	0.383	0.256	10	16	fair	fair
36	Mercer flint, N. D.	11.2	1.5	4.4	–	0.374	0.246	15	9	slight	fair
37	Mercer white cap yellow dent, N. J.	12.2	1.4	4.1	–	0.342	0.231	12	13	fair	fair
38	Clement's white cap, Mich.	12.1	1.4	4.1	–	0.341	0.230	13	11	moderate	severe
39	White flint, R. I.	12.9	1.9	4.5	–	0.418	0.277	13	13	moderate	moderate
40	Moore's white, Wis.	12.5	1.5	4.1	–	0.338	0.301	21	19	none	fair
	Average	11.4	1.4	4.2	–	0.366	0.248	15	13		

finished diet correlates with the variations in degree of rickets produced. In this connection, it may be pointed out that our results do not entirely agree with those reported by Brown and coworkers[7] in which difficulty in producing rickets upon a ration containing more than 0.25 per cent P is indicated. We heartily agree with their thesis that the percentage phosphorus in rachitogenic diets should be stated.

In view of the evidence herewith presented, we conclude that the presence of a significant amount of antirachitic substance definitely affects the degree of rickets produced and causes wide differences in the rachitogenic properties of the corns tested.

It seems to us a reasonable premise that in testing a substance for its antirachitic potency, the animals at the start of the feeding period should be in a condition caused by the absence of the factor which is to be assayed, not in a condition caused by a partial deficiency of this factor. In testing the ability of a substance to cure rickets, the animals should be rachitic, not partially rachitic. Judged by this criterion, if "freshly ground" corn were used by each of forty laboratories which had been supplied with samples of corn identical with ours, at the end of twenty-one days only one laboratory would have animals with severe rickets, 14 laboratories would have animals with moderate rickets, 13 would have animals with fair rickets, 6 would have animals with slight rickets, and 6 would have failed to produce any rickets at all.

Even if these corns had been ground and then stored before using in the diet, although the results would have been favorable in a larger number of cases (12 samples supporting the production of satisfactory 4+ rickets, 11 giving 3+, 14 causing 2+, 2 producing rickets of 1+ degree, and only 1 failing completely to cause a detectable rachitic condition), still it must be evident that this preliminary grinding and storage for six months is merely a palliative and not a remedy and does not relieve the user of the necessity of preliminary assay of his corn sample.

Space prohibits the discussion of the varying rates of growth with the samples tested and whether this has any bearing upon the results. We can find no correlation between our growth curves and the severity of rickets developed and conclude that the relation is negligible if present at all.

The development of a rachitogenic diet more reliable than those containing variable substances such as corn, is necessary. We have inaugurated experiments toward this end.

REFERENCES

1. Harris, R. S., and Bunker, J. W. M.: Note on the Corn Component of a Rachitogenic Diet, Science **73**: 95, 1931.
2. Bourdillon, R. B., Bruce, H. M., Fischmann, C., and Webster, T. A.: Quantitative Estimation of Vitamin D by Radiography, Sp. Report Series No. 158. Med. Res. Council, 1931.
3. Neumann, A.: Ztschr. physiol. Chem. **37**: 129, 1902.
4. Treadwell, F. P., and Hall, W. T.: Analytical Chemistry, ed. 6, **2**: 81, 530.
5. Johnson, C. M.: The Determination of Phosphorus, J. Ind. Eng. Chem. **11**: 113, 1919.
6. Holmes, A. D., and Tripp, F.: Factors Which Influence the Effectiveness of a Rachitogenic Ration (abstract), J. Biol. Chem. **97**: ix, 1932.
7. Brown, H. B., Shohl, A. T., Chapman, E. E., Rose, C. S., and Saurwein, E. M.: The Effect of Various Levels and Ratios of Calcium and Phosphorus in the Diet Upon the Production of Rickets, J. Biol. Chem. **98**: 207, 1932.

THE RELATION OF BLOOD GLUTATHIONE TO THE HEMOGLOBIN AND AMOUNT OF RED CELLS*

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GLUTATHIONE, found widely in plant and animal tissues, is the chief of the sulphydryl compounds which have the reversible property of accepting and giving up hydrogen. "The autoreduction and oxydation of glutathione depend on the simultaneous presence of catalytic metals and an additional factor" (Wurmser), conditions found only in living cells. This faculty constitutes an intracellular respiration. In the blood, glutathione exists only in the cells and might, therefore, be expected to follow their fluctuations. We find, however, that it does not do this, that the amount of glutathione even in severe anemia is usually near the normal level, so that its quantity relative to the bulk of the containing cells is increased. The glutathione is, therefore, independent of the hemoglobin, and is rather connected with the metabolism of the erythrocyte as a living cell. Our work suggests that glutathione is larger in amount proportionately in young cells and a normal glutathione in an anemic blood is evidence of replacement.

To learn the relation, if any, of glutathione to hemoglobin, to number of red cells, cell volume, and to the other reducing substances of the blood, we chose for study a series of anemic bloods. In pathologic bloods the wide deviations of the various findings would throw into light immediately any quantitative connection between any group of the constituents, while with the normal of a biologic substance the narrower limits of normal deviation might place some of the elements examined in a specious relationship expressible by a simple figure and made plausible by statistical correlation. While we were working, there appeared the paper of Woodward and Fry[1] on blood glutathione (GSH) in which they computed Gabbe's quotient, $\frac{\text{GSH}}{\text{R.B.C.}}$, and added one of their own, $\frac{\text{GSH} \times \text{Hb}}{\text{R.B.C.}}$. In 23 bloods Gabbe's quotient ranged from 4.9 to 9.1, the quotient of Woodward and Fry from 5.3 to 8.2. This would hardly prove any relation between these elements in normal blood. Our tables of anemic bloods show still more clearly this lack of quantitative relation.

Glutathione† was determined by direct titration with N 1/1,000 iodine using starch as internal indicator, as advised by Delaville and Kowarski,[2] adding 2 c.c. of 25 per cent KI to 10 c.c. of tungstate filtrate as suggested by Perlzweig and Delrue.[3] The iodine solution was checked by titration of a

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†Glutathione received through the courtesy of Dr. E. M. MacKay.

standard glutathione solution. We used the ordinary 1/10 tungstate blood filtrate. The stability of glutathione in this filtrate will appear in the note on tissue filtrates.

The hemoglobin was determined by the Newcomer method (B. & L.), a method that is practical and is as accurate as the Van Slyke oxygen capacity method when the Newcomer glass plate has been standardized by the latter,[4] and with which duplicate tests check so closely as to be unnecessary. As a percentage we report the hemoglobin by Williamson's normal of 16.92 gm. per 100 c.c. for men, 15.53 gm. for women. This seems to be accurate for our locality, San Diego. The careful work of Osgood and Haskins has shown their normal of 15.8 gm. for men, and 13.7 for women to be correct for Oregon where their work was done, but use of their normals made the color and volume indices too high for the clinical picture in our cases. The wide variation of the red cell count and of the hemoglobin in health makes any standard quite arbitrary. The difference between men and women's blood is really a difference perhaps occasioned by the difference between their muscular activity. Sedentary persons should probably be appraised by the normal female standard.

As volume index, $\text{VI} = \frac{\text{per cent normal cell volume}}{\text{per cent normal cell count}}$, we took 5 million as normal red cell count, and 48 per cent (Sanford[5]) as normal volume of packed cells. We tried out the volume index by the method of Osgood, Haskins and Trotman,[6] $\text{VI} = \frac{\text{per cent cell volume}}{\text{per cent R. B. C.}}$, when the volume of packed red cells per 100 c.c. blood, calculated to a red cell count of 5 million (men 41 per cent, women 43 per cent packed cells) is taken as 100 per cent cell volume for the sex, and when 5 million per c.mm. is taken as 100 per cent red cells, but this index was considerably too high for our locality, giving with the bloods of the secondary anemias in Table I an average of 1.1; with the anemias from hemorrhage, Table II an average of 0.9; with the subnormal bloods in Table IV, the average was 1.1.

The color index was calculated by dividing the percentage of 5 million cells into the percentage of hemoglobin, obtained as above. So many indices have been advised for clinical blood work that it seemed best to choose these two which are better known and simpler. Both are subject to inexplicable variations. The rule is given that if either the color or volume index is over 1.25, the anemia is pernicious. There are many exceptions to this among our cases.

The apparent blood sugar determined by the Folin-Wu method, as is well known, includes a nonglucose sugar in small amount, the glucid X of Fonte's and Thivolle, saccharoid of Benedict, which is not glutathione.[7] The nonglucose reduction beyond that of the Folin-Wu figure, as measured by the Ionesco ferricyanide technic[8] in the same tungstate filtrate is reported in terms of glucose. This, the Y-reduction first reported by Pickard, Pierce, Marsden, Tanaka and Townsend,[9] includes the reduction given by glutathione. One-half the figure for the amount of glutathione in milligrams per 100 c.c. subtracted from the Y-reduction (excess of the Ionesco technic over the Folin-Wu,

as glucose) leaves the variable part of the Y-reduction, due to unknown substances which are greatly increased in certain pathologic conditions. Glutathione is separated from the other part of the Y-reduction by the preparation of unlaked blood filtrates (Folin), it being then precipitated with the red cells.[10]

The bloods are arranged in the tables in order of increasing amounts of glutathione. Table I gives the findings in 23 cases of secondary anemia of

TABLE I

NONHEMORRHAGIC SECONDARY ANEMIAS

PATIENTS REFERRED TO BY NUMBER TOGETHER WITH NAME OF ATTENDING PHYSICIAN		RED CELL COUNT IN MILLIONS	HEMOGLOBIN, GRAMS PER 100 C.C. NEWCOMER HEMOGLOBINOMETER, B. & L.	GLUTATHIONE IN MG. PER 100 C.C.	CELL VOLUME, ACTUAL PERCENTAGE OF CORPUSCLES, WINTROBE HEMATOCRIT.	HEMOGLOBIN IN PERCENTAGE, WILLIAMSON'S NORMALS, 16.92 MALE, 15.53 FEMALE	COLOR INDEX	VOLUME INDEX, OSGOOD	BLOOD SUGAR, FOLIN-WU, MG. PER 100 C.C.	REDUCTION, IONESCO TECHNIC, AS GLUCOSE, MG. PER 100 C.C.	Y-REDUCTION, EXCESS OF IONESCO OVER FOLIN-WU, MG. PER 100 C.C., AS GLUCOSE
1. Oatman	Secondary anemia	4.1	14.5	6.6	40	m86	1.1	1.0	82	102	20
2. Oatman	Diabetes	4.4	13.8	8.8	41	m82	0.9	1.0	145	158	13
3. Ramer	Hodgkin's disease	4.0	11.0	11.5	49	f71	0.9	1.2	124	180	56
4. Molitor	Hypertrophy prostate	3.2	10.8	15.0	–	m64	1.0	–	78	170	92
23. Marsden, Sr.	Abscessed teeth	4.8	12.4	17.4	37	f80	0.8	0.8	94	112	18
5. Doria	Postinfluenza	4.0	12.0	20.0	31	m71	0.9	0.8	85	164	65
6. H. Newton	Aplastic	3.9	14.0	20.0	44	m87	1.1	1.2	107	123	16
7. Ball	Infection	–	9.0	20.0	38	m54	–	–	128	152	24
8. Kennell	Aplastic secondary	3.8	12.2	21.6	35	f78	1.0	1.0	88	126	38
9. Chartres	Typhoid	4.3	8.3	24.1	40	f55	0.6	1.0	180*	241	61
10. Alberty	Neurosis	4.2	15.0	24.6	45	m89	1.0	1.1	93	124	31
11. Ratty	Diabetes	4.2	12.8	25.0	42	f82	1.0	1.0	145	200	55
12. Miller	Diabetes	3.2	9.6	26.5	35	m57	0.9	1.1	260	380	120
13. Hileman	Optic atrophy	4.4	11.2	27.0	40	f72	0.8	0.9	85	162	77
14. Ball	Hyperthyroidism	2.9	5.4	28.0	22	f35	0.6	0.8	–	–	–
15. Eager	Infection	3.9	12.3	28.3	29	f79	1.0	0.8	105	190	85
16. Molitor	No. 4, 3 weeks later	4.6	11.8	29.2	32	m70	0.8	0.7	95	145	50
17. Molitor	No. 4, 2nd week, operated	3.1	13.0	33.0	29	m77	1.2	1.1	101	276	175
18. Kennell	Sinusitis	3.5	12.8	34.6	41	f82	1.2	1.2	110	160	50
19. Fox	Diabetes	3.8	12.8	35.4	34	m76	1.0	0.9	87	117	30
20. Kerch	Postinfluenza	5.1	13.2	37.2	41	m78	0.8	0.8	92	136	44
21. Worthington	Neurosis	4.0	13.5	39.2	40	m80	1.0	1.0	117	204	81
22. Potter	Aplastic secondary	4.0	12.2	40.1	31	f78	0.9	0.8	100	150	50
	Averages	3.9	11.9	24.9	37	73%	0.9	1.0			

*After intravenous glucose.

either hemolytic or aplastic type. The glutathione is normal except in four cases and in these bloods the anemia is not particularly marked, but in all four the diseased condition is of several years' duration. Table II lists 10 cases of anemia from hemorrhage. The patient in Case 31 had a glutathione of 4.2 mg.; a week later (blood 40), with a rise in the red cells from 1.6 to 2.5 millions, the gluta-

thione was normal, 30 mg. per 100 c.c. Assuming 30 mg. glutathione and 5 million cells as normal, the concentration of glutathione in samples 31 and 40 is respectively 50 per cent and 200 per cent of the normal concentration. Calculated to a normal cell volume of 46 per cent the glutathione concentration is 64 per cent (31) and 250 per cent (40). In the erythrocytes put in circulation immediately after hemorrhage, the glutathione was exceptionally low. Its restoration to normal in a week would seem indicative of its importance. The same drop after hemorrhage occurred in Case 51 (Table VI). Unfortunately we were unable to obtain follow-up specimens on other patients in Table II. In these

TABLE II

POSTHEMORRHAGIC ANEMIAS

PATIENTS REFERRED TO BY NUMBER TOGETHER WITH NAME OF ATTENDING PHYSICIAN		RED CELL COUNT IN MILLIONS	HEMOGLOBIN, GRAMS PER 100 C.C. NEWCOMER HEMOGLOBINOMETER, B. & L.	GLUTATHIONE IN MG. PER 100 C.C.	CELL VOLUME, ACTUAL PERCENTAGE OF CORPUSCLES, WINTROBE HEMATOCRIT.	HEMOGLOBIN IN PERCENTAGE, WILLIAMSON'S NORMALS, 16.92 MALE, 15.53 FEMALE	COLOR INDEX	VOLUME INDEX, OSGOOD	BLOOD SUGAR, FOLIN-WU, MG. PER 100 C.C.	REDUCTION, IONESCO TECHNIC, AS GLUCOSE, MG. PER 100 C.C.	Y-REDUCTION, EXCESS OF IONESCO OVER FOLIN-WU, MG. PER 100 C.C., AS GLUCOSE
31. Ball	Abortion	1.6	4.1	4.2	10	f26	0.8	0.7	89	155	61
32. Ball	Intestinal hemorrhage	0.7	2.3	9.0	6	m16	1.4	0.9	160*	174	14
33. Ball	Obstetric	2.2	5.8	11.5	32	f37	0.8	1.5	87	126	39
34. Ball	Obstetric	1.9	5.5	11.6	12	f35	0.9	0.7	–	72	–
35. Ball	Abortion	3.3	7.5	12.5	20	f48	0.7	0.6	118	154	36
36. Ball	Duodenal ulcer	2.4	7.3	13.0	20	m43	0.9	0.9	87	119	32
37. Ball	Carcinoma of sigmoid	1.8	5.5	21.2	7	f53	0.9	0.4	95	112	17
38. Ball	Obstetric	2.5	6.5	21.8	21	f42	0.8	0.9	70	105	35
39. Crawford	Hemorrhage of intestine	1.9	5.9	21.9	15	f38	1.0	0.8	110	160	50
40. Ball	31, week later	2.5	6.2	30.0	18	f40	0.8	0.8	90	120	30
41. Oatman	Hemorrhage fibroid	4.2	10.2	31.5	32	f66	1.0	0.8	118	–	–
	Averages	2.3	6.1	17.1	17.5	40	0.9	0.8			

*After intravenous glucose.

bloods of anemia from hemorrhage the glutathione follows somewhat the loss in cells and hemoglobin. In nearly all the bloods in Table I, the glutathione is in the normal range of 20 to 40 mg., average 30 mg., and its importance to the metabolism of the erythrocyte can be deduced from this together with the absence of any ratio between the amount of glutathione and the number of cells, amount of hemoglobin, cell volume percentage, or the amount of the other reducing bodies. Graphs drawn experimentally to trace any trend or grouping of the GSH and other blood findings showed only the widest divergence. We believe that the tabulated results bear out the idea that the hemoglobin and cell volume percentage should be reported in their actual amounts,

as are the red cell count and the blood chemical findings, rather than as a percentage of normal averages, which latter, like the indices, can be misleading. The clinician should base his diagnosis on findings referred to the normal range for the individual type. Ratios between the findings not apparent on inspection are apt to be misleading or unimportant.

Table III gives the glutathione in 7 cases of anemia diagnosed as pernicious; this is not enough from which to draw any conclusions but the low glutathione is noticeable. The bloods in Table IV are mostly in the lower limit of normal but were placed there because of apparent good health. None of the eight were doing any regular active muscular work. Except the patient in

TABLE III

PERNICIOUS ANEMIAS

PATIENTS REFERRED TO BY NUMBER TOGETHER WITH NAME OF ATTENDING PHYSICIAN		RED CELL COUNT IN MILLIONS	HEMOGLOBIN, GRAMS PER 100 C.C. NEWCOMER HEMOGLOBINOMETER, B. & L.	GLUTATHIONE IN MG. PER 100 C.C.	CELL VOLUME, ACTUAL PERCENTAGE OF CORPUSCLES, WINTROBE HEMATOCRIT.	HEMOGLOBIN IN PERCENTAGE, WILLIAMSON'S NORMALS, 16.92 MALE, 15.53 FEMALE	COLOR INDEX	VOLUME INDEX, OSGOOD	BLOOD SUGAR, FOLIN-WU, MG. PER 100 C.C.	REDUCTION, IONESCO TECHNIC, AS GLUCOSE, MG. PER 100 C.C.	Y-REDUCTION, EXCESS OF IONESCO OVER FOLIN-WU, MG. PER 100 C.C., AS GLUCOSE
45. Ball	Pernicious anemia 7 years	0.6	2.0	1.7	6	f12	1.0	1.0	145	156	11
46. Austin	Treated case	–	11.3	11.7	33	–	–	–	–	–	–
47. Alberty	Occasionally treated	3.2	12.5	13.2	30	m74	1.2	1.2	122	146	24
48. Doria	Pernicious anemia 8 years	2.6	12.0	18.1	34	m71	1.2	1.4	100	105	5
50. Ball	Treated	1.0	5.6	27.7	9	f37	1.8	0.9	89	130	41
51. Marsden	Untreated	3.2	12.5	28.6	41	f82	1.2	1.3	85	191	106
52. Roberts	Untreated	3.3	13.0	–	43	f84	1.3	1.3	80	–	–
52. Month later	Treatment	4.4	12.2	34.2	35	78	0.9	0.8	81	128	47

Case 61 all were regular users of alcohol, the distinction of 62 and 64 as "alcoholic" was because they were exceptional in the amount consumed daily, the patient in Case 64 using 300 c.c. (as absolute alcohol) daily, and it was thought that this might influence the blood sugar, in both cases at the upper normal limit.

We were able to follow two cases. Case I was a young man, a college student in poor health in that he easily became exhausted. Physical examination was negative except for a basal metabolic rate of minus 16. His father died of Hodgkin's disease. While his blood smears were negative, the high color and volume indices suggest pernicious anemia. The blood glutathione remained low (Table V). There is evidently a severe metabolic disturbance.

The low basal rate may be due to the low glutathione and resultant lower intracellular metabolism and not to the thyroid.

Case 51, a woman in midlife, was in a run-down condition, asthenic, nervous, worried and suffering from insomnia at the time of the first examination when the Y-reduction was high, 106 mg. The high blood indices suggested pernicious anemia; there were no nucleated cells. The second examination was like the first except that the patient was feeling stronger and sleeping well and the Y-reduction was then normal, 13 mg., of which glutathione accounted for 12 mg. The high red count with lower hemoglobin on December 6 (Table VI) was due to severe menstrual loss; she had been on liver and iron treatment for a week. The glutathione fell to 8.7 mg. with the hemorrhage, and the color index from 1.4 to 0.9.

TABLE IV

NORMAL

PATIENTS REFERRED TO BY NUMBER	RED CELL COUNT IN MILLIONS	HEMOGLOBIN, GRAMS PER 100 C.C. NEWCOMER HEMOGLOBINOMETER, B. & L.	GLUTATHIONE IN MG. PER 100 C.C.	CELL VOLUME, ACTUAL PERCENTAGE OF CORPUSCLES, WINTROBE HEMATOCRIT.	HEMOGLOBIN IN PERCENTAGE, WILLIAMSON'S NORMALS, 16.92 MALE, 15.53 FEMALE	COLOR INDEX	VOLUME INDEX, OSGOOD	BLOOD SUGAR, FOLIN-WU, MG. PER 100 C.C.	REDUCTION, IONESCO TECHNIC, AS GLUCOSE, MG. PER 100 C.C.	Y-REDUCTION, EXCESS OF IONESCO OVER FOLIN-WU, MG. PER 100 C.C., AS GLUCOSE
61. Age over 60	4.6	12.0	12.3	38	f78	0.9	0.9	124	134	10
62. Alcoholic	4.7	15.0	18.4	44	m89	1.0	1.0	115	138	23
63. Aged 50	5.4	16.4	18.4	45	m98	0.9	0.9	112	152	40
64. Aged 50, alcoholic	4.5	14.0	22.0	41	m83	0.9	1.0	120	147	27
65. Student	4.1	16.2	24.6	45	m96	1.2	1.1	85	108	23
66. Student	4.7	15.4	28.3	41	m92	1.0	0.9	95	109	14
67. Student	5.6	15.5	31.7	46	m92	0.8	0.9	101	132	31
68. Sedentary	5.1	16.0	37.5	41	m94	0.9	0.8	81	112	31
Averages	4.8	15.1	24.1	43	91	0.9	0.9			25

The Y-reduction was originally calculated as a difference in reduction between different filtrates and reduction technics, and was then found to average about 40 mg. per 100 c.c. in normal blood.[9] There have not been many normals examined since the use of the Ionesco technic as a comparison with the Folin-Wu in the same tungstate filtrate. As Fabre[11] says, the difficulty of dissolving the sulphydryl derivatives tends to demonstrate that reduced glutathione does not exist in a free state, but in a potential form in the cells of the organism. This is borne out in that copper solutions are not reduced by the glutathione in the blood, while glutathione added to blood before precipitation gives an added reduction with the Folin-Wu (20 per cent as glucose) and Ionesco technics (50 per cent, as glucose) and is also quantitatively recovered

by the iodine titration and in the increase in nonprotein nitrogen.[10] The Ionesco technic shows the reduction of true glucose and of glucid X, as does the Folin-Wu, and in addition the reduction of the glutathione of the cells together with a reduction due to unknown substances.[10] We believe from the present work that the normal fasting Y-reduction nearly approximates that part of the Y-reduction due to glutathione. Certainly a Y-reduction of over 25 mg. shows an increase in the part due to the unknown substances which are increased, or retained, especially in kidney disease.

The possibility that the quantity of glutathione in a tissue might serve as an index to its metabolic activity is suggested by the work of Blanchetière

TABLE V

FOLLOW-UP DATA ON TWO CASES

PATIENTS REFERRED TO BY NUMBER	RED CELL COUNT IN MILLIONS	HEMOGLOBIN, GRAMS PER 100 C.C. NEWCOMER HEMOGLOBINOMETER, B. & L.	GLUTATHIONE IN MG. PER 100 C.C.	CELL VOLUME, ACTUAL PERCENTAGE OF CORPUSCLES, WINTROBE HEMATOCRIT.	HEMOGLOBIN IN PERCENTAGE, WILLIAMSON'S NORMALS, 16.92 MALE, 15.53 FEMALE	COLOR INDEX	VOLUME INDEX, OSGOOD	BLOOD SUGAR, FOLIN-WU, MG. PER 100 C.C.	REDUCTION, IONESCO TECHNIC, AS GLUCOSE, MG. PER 100 C.C.	Y-REDUCTION, EXCESS OF IONESCO OVER FOLIN-WU, MG. PER 100 C.C., AS GLUCOSE
Case 1. Nov. 16	4.1	14.5	6.6	40	m86	1.0	1.0	82	102	20
Dec. 6	4.3	12.8	7.3	43	76	0.8	1.0	80	92	12
Jan. 5	3.7	12.5	7.0	40	74	1.6	1.1	100	110	10
Feb. 3	4.5	12.8	5.2		76	0.7		–	–	–
Case 51. Oct. 28	3.2	12.5	28.6	41	f82	1.2	1.3	85	191	106
Nov. 7	3.0	12.8	23.8	42	83	1.4	1.5	89	102	13
Dec. 6*	4.0	11.5	8.7	42	75	0.9	1.1	100	114	14
Jan. 5	4.0	12.5	25.4	43	81	1.5	1.1	110	136	26

*During severe menstrual hemorrhage.

and Mélon, cited by Wurmser,[12] using dog and rabbit tissues. Mme. Boucher-Firly found that the total amount of glutathione in eels diminished as they grew older, and there is a parallelism between the amount of glutathione and the oxygen consumption.[13] Murray found the same progressive diminution in glutathione in chicken embryo. His work is cited by M. Labbé, H. Labbé and Nepveux in a résumé of the work done on the clinical aspect of the glutathione content of blood and other tissues.[14] They state that neither fasting nor diet change the glutathione in the blood nor is there any connection between the quantity of glutathione and changes in the basal metabolism. They think that "the mechanism of the action of glutathione is extremely complex, that it is probable that the sulphydryl bodies act at the level of certain stages of the degradation of acid chains where there is no immediate or simultaneous

effect on the terminal stages such as glucose, urea or uric acid.'' They found a low blood glutathione in many patients with disturbed nutrition: gout, diabetes, cirrhosis, obesity, half or less of the 21 mg. per cent which they find in normal blood, following the technic of Tunnicliffe modified by Randoin and Fabre.

That the comparison with the normal glutathione of the tissue may be an index to the restorative capacity in pathologic conditions is suggested by our tables of anemic bloods. Table VI gives the glutathione content in cavy and human tissues, including three cancers. We macerated the tissues in distilled water for an hour, then precipitated with tungstic acid to obtain a 1/10 filtrate as with blood. There has been some criticism of the tungstate extraction of glutathione. Following Tunnicliffe most of the work with glutathione has been done on triochloracetic acid filtrates. Repeated tests on our tissue filtrates either alone or standing on the tissue and continuing the ex-

TABLE VI

GLUTATHIONE IN TISSUES (FIGURES, MILLIGRAMS PER 100 GM.)

1. Guinea pig—muscle	36.9	5. Human—papillomas from ovarian cyst	145.0
2. Guinea pig—		6. Human—cancer breast	
muscle	54.6	mass under skin	220.0
liver	397.0	deep in breast	336.0
(pregnant) uterus	170.0	same mass deep in breast	329.0
placenta	81.6	pectoral muscle	131.0
3. Guinea pig—		same muscle	127.0
muscle	50.7	fat	0.9
same, after 2 weeks' extraction	50.6	7. Human—cancer breast, recurrent	140.8
4. Guinea pig embryo—		8. Human—fibrous mastitis	1.9
muscle	54.6	9. Human—	
liver	170.0	uterine muscle	125.9
		decidua	45.5
		10. Human—embryonal cancer testis	475.0

traction, checked so closely after hours and even days that we give the result of one test in which the extraction continued two weeks and the glutathione determination was the same within the limits of error. The tungstate extraction seems to be immediate and complete. For these reasons we do not think the criticism of tungstate filtrates for glutathione are valid. We tried the sulphosalicylic acid filtrate advised by Woodward and Fry but did not get a distinct end-point with the iodate titration, and the results were lower than with tungstate and iodine.

With guinea pig liver and striated muscle our results correspond with those obtained by Blanchetière and Mélon. In the embryo cavy the liver is a tremendous storehouse for glycogen, nearly 2.8 per cent as glucose. The fetal liver has a low glutathione content compared to that of the adult liver when the digestive function is active. The glutathione in the pregnant uterine muscle of the cavy was much higher than that in the smooth muscle examined by the French investigators. The highest glutathione we found was in an embryonal carcinoma of the testis, and except for liver the largest amount

found in other tissues was in parts of an actively growing cancer of the breast. This is in accord with the findings of Thompson and Voegtlin (cited by Wurmser). Their statement that these tumors seem to collect glutathione and that it diminishes in the rest of the organism is contradicted by Labbé and Nepveux, and for the blood at least is not sustained by Woodward and Fry, who found a normal blood glutathione in their six cancer cases. In the embryonic carcinoma of the testicle, cancer of the breast, papilloma and other tissues the area in the microscopic picture occupied by nuclei is in direct ratio with the glutathione content of the tissue, suggesting that this substance is chiefly in the nuclear protoplasm. Fat, containing almost no cytoplasm, had the lowest glutathione, almost as low was given by the dense white fibrous tissue from a chronic mastitis. Tissue activity and the amount of glutathione seem to correlate to such an extent that the latter may be taken as an index to intracellular metabolism.

SUMMARY

The blood glutathione is necessarily lowered when there is a sudden loss of blood, but its amount is early restored to the normal preceding that of the number of red cells and the hemoglobin. There is no quantitative relation between the hemoglobin and the glutathione of the blood. Although the glutathione of the blood is part of the content of the red cells, its quantity does not follow variations in either the cell count or the cell volume. The average amount of glutathione in 2 patients with nonhemorrhagic secondary anemia (Table I) was 24.9 mg.; excluding the 5 bloods with glutathione under 20 mg., the average was 28.5 mg. For the bloods taken after severe hemorrhage (Table II) the average glutathione was 17.1 mg., average red count 2.3 million, average cell volume 17.5 per cent. Calculated to a red count of 5 million, the glutathione would be 37 mg., a 23 per cent excess concentration in the cells, and to a normal cell volume of 46 per cent, there is a 150 per cent glutathione concentration. This gives added emphasis to the findings in Case 1, Table V. In this patient the glutathione as calculated for 5 million cells is 8.0, 8.5, 9.5, 5.8 mg. for the four examinations, averaging only 27 per cent of the normal concentration as to cell count, and 26 per cent of the normal as to cell volume. The persistently low glutathione in the blood of this patient, asthenic, but without definite physical findings, constitutes a deficiency in cell protoplasm new to medical literature.

REFERENCES

1. Woodward, G. E., and Fry, E. G.: The Determination of Blood Glutathione, J. Biol. Chem. **97**: 465, 1932.
2. Delaville, M., and Kowarski, L.: Étude de l'indice de l'iode du sang, Bull. Soc. Chimie Biol. **14**: 660, 1932.
3. Perlzweig, W. A., and Delrue, G.: The Use of the Starch Iodine End Point in Tunnicliffe's Method for the Determination of Glutathione in Tissues, Biochem. J. **21**: 1416, 1927.
4. Pickard, R. J., Pierce, L. F., and Worthington, R. L.: On Hemoglobinometry, J. Lab. & Clin. Med. **15**: 746, 1930.
5. Todd and Sanford: Clinical Diagnosis by Laboratory Methods, ed. 7, Philadelphia, 1931, W. B. Saunders Co.

6. Osgood, E. E., Haskins, H. D., and Trotman, F. E.: The Value of Accurately Determined Color, Volume and Saturation Indexes in Anemias, J. LAB. & CLIN. MED. 17: 859, 1932.
7. Pickard, R. J.: Trois corps réducteurs du sang, Bull. Soc. Chimie Biol. 14: 598, 1932.
8. Pickard, R. J., and Godwin, F. W.: The Nonglucose Reducing Bodies in Diabetic Blood, J. LAB. & CLIN. MED. 18: 3, 1932.
Pickard, R. J.: The Separation from Blood Glucose of Two Non-glucose Reducing Substances, Am. J. Clin. Path. 2: 255, 1932.
9. Pickard, R. J., Pierce, L. F.: Marsden, C. S., Jr., Tanaka, R. K., and Townsend, H. A.: A Nonglucose Reduction Present in Normal and Increased in Nephritic Blood, J. LAB. & CLIN. MED. 17: 471, 1932.
10. Pickard, R. J.: La part du glutathione dans la réduction, Y. Bull. Soc. Chimie Biol. 15: 781, 1933.
11. Fabre, R., and Simonnet, H.: Contribution à l'étude du pouvoir oxydoréducteur des tissus, Bull. Soc. Chimie Biol. 12: 777, 800, 1930.
12. Wurmser, R.: Oxydations et réductions. Les Presses Universitaires de France, Paris, 1930.
13. Boucher-Firly, S.: Le glutathione chez l'anguille et le congre, Compt. rend. Soc. de Biol. 112: 1921, 1933.
14. Labbé, M., Labbé, H., and Nepveux, F.: Techniques de laboratoire appliquées aux maladies de l'appareil digestif et de la nutrition, Masson et Cie., Paris, 1932 (p. 849, seq.).

THE INACTIVATION OF GROWTH HORMONE*

II. As a Result of Exposure to Air

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IN THE course of experiments dealing with the effect of growth hormone upon the brain-weight-body weight ratio[1] it was noted that in order to maintain continuous body growth in rats (Mus norvegicus albinus) it was necessary to use fresh extract almost every other week. This became necessary because after an initial growth stimulating effect, it was noted that the extract soon lost its potency. It therefore became quite an interesting problem to determine the factors causing this loss of potency of the extract and recently this laboratory reported improper refrigeration to be one of these.[2]

As a result of this, it was found advantageous to place a few drops of phenolphthalein as an indicator into each bottle of growth hormone and to use the extract only as long as the P_H was maintained at approximately 8.3. The addition of phenolphthalein led to a rather interesting observation; namely, that pouring the extract into a beaker prior to administration caused a fading out of the red color with a consequent loss of alkalinity. At first this phenomenon was believed to be due to acid fumes in the air since our laboratory is situated in an industrial center.

In order to rule out such an atmospheric factor, 30 c.c. of growth hormone were placed in an open beaker within a refrigerator whose temperature was kept at 10° C. In another beaker, were placed 30 c.c. of meat extract which is ordinarily used for control injections. The alkalinity of each extract was just sufficient to give a pink tinge to phenolphthalein (P_H 8.3). These were

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then allowed to stand, thus exposed to the cold air of the ice box, for twenty-four hours, at the end of which time, the growth hormone solution was completely decolorized, while the meat extract maintained its color. The only single factor which could possibly have decreased the alkalinity of the growth hormone was air, since refrigeration was adequately maintained. Furthermore, as long as the extract was kept stoppered no diminution in its alkalinity could be noted.

If allowed to stand long enough, this alkalinity will be decreased even to the point of becoming truly acidic, since Morrell[3] checking up on some of our findings noted in one case a drop in P_H from 8.3 to 6.76.

The necessity for keeping the solutions well stoppered even during injections becomes quite apparent since, as in the case where inactivation is the result of inadequate refrigeration, growth effects can be obtained only with definitely alkaline material.

CONCLUSIONS

1. Growth hormone is active only so long as it is alkaline.
2. Alkalinity is diminished and even lost when the hormone is exposed to the air.

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REFERENCES

1. Rubinstein, H. S.: The Effect of Growth Hormone on the Brain-Weight-Body Weight Ratio, Anat. Rec. **53**: 265, 1932.
2. Rubinstein, H. S.: The Inactivation of Growth Hormone as a Result of Inadequate Refrigeration, J. Lab. & Clin Med. **19**: 63, 1933.
3. Morrell, J. A.: Personal communication.

LABORATORY METHODS

THE DETERMINATION OF HEMOGLOBIN BY THE IRON CONTENT METHOD*

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THE presence of iron in blood was first demonstrated by Menghini of Bologna in 1747,[1] and for more than a century the study of this problem has excited much interest. Quantitative analyses were made by Denis in 1830,[2] and quite accurate estimations were accomplished by others before 1850. The early investigators obtained a remarkable uniformity of results[3] by working with relatively large amounts of blood.

After the isolation of hemoglobin in crystalline form by Funke in 1851,[4] analyses of hemoglobin crystals by Hoppe-Seyler[5] showed sufficient iron in the crystal to account for all the iron found in whole blood. In the first monograph on hemoglobin, published in 1871,[6] Preyer compared the quantitative determination of hemoglobin by the spectroscopic, iron content, and colorimetric methods and found that they gave identical results. In 1877,[7] Malassez' first paper on clinical methods for estimating hemoglobin described three chemical methods, by determination of the iron content, of the oxygen capacity, and of the hematin content of the blood. Even at this early date in clinical hemoglobinometry, however, the colorimetric method already was well established and numerous procedures were in use. The iron content method was little used for many years, although the ferrometer described by Jolles in 1898[8] was employed to some extent and papers on the iron content of the blood appeared from time to time.

In recent years, there has been much improvement in the methods for estimating hemoglobin and renewed interest in the two available accurate chemical methods. This has been due largely to the development by Wong[9] of a simple and precise microchemical method for the determination of iron and also to Van Slyke's[10] development of a blood gas apparatus in which oxygen capacity of the blood can be determined correctly by using small quantities of blood. Laboratory workers agree that all hemoglobinometers should be standardized by some accurate chemical method, and the oxygen capacity method has so far been the one preferred. Blood gas analyses, however, require experience and special apparatus. The determination of iron is a simpler procedure, and if it yields results as accurate as the measurement of hemoglobin by the oxygen capacity method, it should be of equal value as a method of standardization and

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also should be available for special laboratory estimations of hemoglobin. If the estimations of hemoglobin by the iron content and by the oxygen capacity methods check within the limit of error, it is apparent that the ratio must be a constant one and that the iron content is an accurate measure of the amount of hemoglobin present. For all practical purposes, the iron content of the whole blood may be considered an indicator of the hemoglobin just as the oxygen capacity is taken as a measure of hemoglobin.

The oxygen capacity method determines all the hemoglobin present in blood except the small amount combined with carbon monoxide. Gettler[11] has shown

TABLE I

COMPARISON OF HEMOGLOBIN DETERMINATIONS BY OXYGEN CAPACITY AND IRON CONTENT METHODS

OBSERVER	NUMBER OF DETERMINATIONS	METHOD	HEMOGLOBIN	
			IRON CONTENT	OXYGEN CAPACITY
Lindsay, Rice and Sellinger	7	Original Wong	Gm. per 100 c.c.	Gm. per 100 c.c.
			7.89	7.88
			8.13	9.06
			12.48	12.21
			14.85	14.15
			15.87	15.16
			15.79	15.89
			18.80	18.78
	Mean		13.40	13.30
Kennedy	8	Kennedy	12.55	12.45
			13.20	12.80
			14.70	14.52
			17.00	16.90
			17.42	17.38
			17.96	17.90
			19.70	19.60
			20.10	20.40
	Mean		16.58	16.49
Haden	10	Dupray modification of original Wong	12.50	12.40
			12.95	13.28
			12.98	13.15
			13.32	13.43
			13.46	13.13
			14.63	14.95
			14.68	15.02
			14.68	15.11
			16.25	15.82
			17.53	17.31
	Mean		14.30	14.36
Haden	11	New Wong method	6.2	6.0
			7.8	7.6
			8.4	8.0
			9.4	9.4
			10.3	10.6
			10.7	10.4
			11.8	11.2
			11.8	12.1
			12.1	12.4
			14.4	14.4
			15.4	15.7
	Mean		10.2	10.2
	Mean of 36 determinations		13.38	13.34

that the carbon monoxide content of the blood is greater in city than in country dwellers but is never sufficient to interfere with clinical determinations of hemoglobin. Riecker[12] found a small amount of iron (1.1 ± 0.022 mg.) in the blood plasma of normals. The remainder of the iron present in blood is in the hemoglobin molecule. In a recent article Reich and Tiedeman[13] have disputed the value of the iron content of the blood as a measure of hemoglobin and have concluded that the ratio between hemoglobin and iron is not constant; they used in their study a colorimetric estimation of hemoglobin.

From time to time over several years I have determined the iron content and the oxygen capacity of specimens of oxalated blood. Lindsay, Rice and Sellinger,[14] and Kennedy[15] have made similar studies. The values for hemoglobin, when calculated in these two ways, have always checked closely and well within the limit of error. I have compiled the data so far available in Table I. In 36 determinations, the mean hemoglobin by the iron content method is 13.38 gm. and by the oxygen capacity method, 13.34 gm. These results indicate that there is no variation in the iron hemoglobin ratio not accounted for by errors in estimation, and prove that the iron content is as accurate a measure of the hemoglobin as is the determination of the oxygen capacity.

My early determinations were made by the Dupray[16] modification of the original Wong method. In this procedure, perchloric acid is added to facilitate digestion and nitric acid to prevent fading of the iron sulphocyanate. Kennedy[15] suggested the digestion of the blood with perchloric and sulphuric acids, and subsequent extraction of the digested material with sodium sulphocyanate and amyl alcohol to prevent fading. In Wong's new method,[17] the iron is split off from the hemoglobin with sulphuric acid and potassium persulphate, and the interfering proteins are precipitated with tungstic acid, and sodium sulphocyanate is added to the filtered solution. The potassium persulphate prevents the fading of the iron sulphocyanate for a long time. I have found no difference in the results obtained by different methods. The new method of Wong[17] is as follows:

REAGENTS REQUIRED

"All the reagents except the standard must be iron-free as shown by blank tests.

"1. Concentrated Sulphuric Acid.

"2. Sodium Tungstate.—Dissolve an appropriate amount of good grade sodium tungstate to make a 10 per cent solution.

"3. Saturated Potassium Persulphate.—Introduce into a small glass-stoppered bottle about 7 gm. of pure potassium persulphate and shake up with 100 c.c. of distilled water. The undissolved portion settles on the bottom to make good any decomposition upon standing.

"4. Potassium Sulphocyanate.—Prepare approximately a 3 N solution by dissolving 146 gm. of pure potassium sulphocyanate in distilled water to make 500 c.c. Filter, if necessary. Add 20 c.c. of pure acetone to improve its keeping quality.

"5. Standard Iron Solution.—Weigh accurately 0.7 gm. of crystallized ferrous ammonium sulphate and dissolve in about 50 c.c. of distilled water. Add to the solution 20 c.c. of dilute (10 per cent) iron-free sulphuric acid, warm slightly, and then add 0.1 N (approximate) potassium permanganate solution to oxidize the ferrous salt completely. Dilute with distilled water to one liter exactly. Each cubic centimeter will contain 0.1 mg. of iron for use as a regular standard. To make weaker standards dilute this standard solution accordingly.

PROCEDURE

"Transfer accurately with an Ostwald pipette 0.5 c.c. of blood into a 50 c.c. volumetric flask and introduce 2 c.c. of iron-free concentrated sulphuric acid. Whirl the flask to agitate the mixture for one or two minutes. Add 2 c.c. of saturated potassium persulphate solution and shake. Dilute to about 25 c.c. with distilled water and add 2 c.c. of 10 per cent sodium tungstate solution. Mix. Cool to room temperature under the tap and then dilute to volume with distilled water. Stopper the flask and invert two or three times to effect thorough mixing. Filter through a dry filter paper into a clean, dry receiving vessel. Pipette exactly 20 c.c. of the clear filtrate into a large test tube graduated at 20 and 25 c.c.

"Measure into another similar test tube exactly 1 c.c. of the standard iron solution containing 0.1 mg. of Fe per c.c. Add with a graduated 1 c.c. pipette 0.8 c.c. of iron-free concentrated sulphuric acid and dilute to the 20 c.c. mark with distilled water. Cool to room temperature under the tap. Now add to both the unknown and standard 1 c.c. of saturated potassium persulphate and 4 c.c. of 3 N potassium sulphocyanate solution. Insert a clean rubber stopper, mix, and compare in a Duboscq colorimeter.

"Calculation.—As the 20 c.c. of filtrate taken represent 0.2 c.c. of the original blood, and the quantity of standard solution used contains 0.1 mg. of Fe, if the reading is made with the standard set at 20 mm., then 20 divided by the reading (r) of the unknown and multiplied by 50 will give the number of mg. of Fe in 10 c.c. of the blood examined. To obtain the percentage of hemoglobin, divide this number by 3.35, since hemoglobin contains 0.335 per cent of iron.

$$\frac{20}{R} \times 50 = \text{mg. of iron per hundred cubic centimeters of blood.}$$

$$\frac{20 \times 50}{R \times 3.35} = \text{percentage of hemoglobin in blood.}"$$

In some of the recent articles on blood iron the results are expressed as iron in milligrams per hundred cubic centimeters rather than as hemoglobin. Murphy, Howard, and Lynch[3] have described an "iron index" in which the relation of the iron in milligrams to the red cell count in millions is calculated. Since, as I have indicated above, for every practical purpose, all the iron present is in the form of hemoglobin, I can see no reason for recording the results in terms of iron rather than of hemoglobin. Likewise, an "iron index" is the same as a color index since the relation of the iron to the cell count is again only another way of expressing the relation of the hemoglobin to the cell count.

Reich and Tiedemann[13] have suggested an "iron-volume index" which expresses the relation of the iron to the mass of packed red corpuscles. Since here again, the iron is really a measure of the hemoglobin, this is really a saturation index and serves only to confuse the issue by multiplying the various indices.

CONCLUSIONS

The iron content method is a simple and accurate method for determining the amount of hemoglobin in the blood, and the results check closely with those of the oxygen capacity method.

The iron method for the determination of hemoglobin deserves wider use in clinical laboratories for the checking of hemoglobinometers and other purposes.

For every practical purpose all the iron present in the blood is in the form of hemoglobin, so the results should be calculated as hemoglobin rather than as "blood iron."

The "iron index" is only another way of expressing *the color* index, and the "iron volume index" another way of expressing the *saturation* index.

REFERENCES

1. Menghini: De ferrarum particulorum sede in sanguine, 1747. Quoted by Fowell. Quart. J. Med. **6**: 179, 1912-13.
2. Denis, P. S.: Rechèrchés experimentales sur le sang humain considère l'état sain, 1830. Quoted by H. Nasse in Das Blut, Bonn, 1836, p. 115.
3. Murphy, W. P., Lynch, R., and Howard, I. M.: The Value of Determinations of the Iron Content of Whole Blood, Arch. Int. Med. **47**: 883, 1931.
4. Funke, O.: Über Milzevenenblut, Ztschr. f. rat. Med., N.F. **1**: 172, 1851.
5. Hoppe-Seyler, F.: Beiträge zur Kenntniss des Blutes des Menschen und der Wirbelthiere. Med.-chem. Untersuch. a. d. Lab. . . . zu Tübing **1**: 169, 1867-1871.
6. Preyer, W.: Die Blutkrystalle, Jena, 1871.
7. Mallassez, L.: Sur les diverses méthodes de dosage de l'hémoglobine et sur un nouveau colorimètre, Arch. de Physiol. norm. et path. **4**: 1, 1877.
8. Jolles, A.: Apparat zur quantitativen Bestimmung des Bluteisens für klinische Zwecke, Deutsche med. Wchnschr. **24**: 104, 1898.
9. Wong, S. Y.: Colorimetric Determination of Iron and Hemoglobin in Blood, J. Biol. Chem. **55**: 421, 1923.
10. Van Slyke, D. D.: Studies of Acidosis. II. A Method for the Determination of Carbon Dioxide and Carbonates in Solution, J. Biol. Chem. **30**: 347, 1917.
11. Gettler, A. O., and Mattice, M. R.: The "Normal" Carbon Monoxide Content of the Blood, J. A. M. A. **100**: 92, 1933.
12. Riecker, H. H.: Iron Metabolism in Pernicious and in Secondary Anemia, Arch. Int. Med. **46**: 458, 1930.
13. Reich, Carl, and Tiedemann, V. G.: A Study of "Iron-Volume Index" of the Blood and Its Significance in the Treatment of Anemia, Am. J. M. Sc. **184**: 637, 1932.
14. Lindsay, J. W., Rice, E. C., and Sellinger, M. A.: A Plea for a Standardized Method of Estimating and Reporting Hemoglobin Values, J. LAB. & CLIN. MED. **11**: 737, 1926.
15. Kennedy, R. P.: Quantitative Determination of Iron in Tissues, J. Biol. Chem. **74**: 385, 1927.
16. Dupray, M.: A Colorimetric Method for the Determination of Iron and Hemoglobin in the Blood, J. LAB. & CLIN. MED. **12**: 917, 1927.
17. Wong, S. Y.: Colorimetric Determination of Iron and Hemoglobin in Blood, J. Biol. Chem. **77**: 409, 1928.

OBSERVATIONS UPON THE LIPOKRIT METHOD FOR THE DETERMINATION OF THE LIPOID CONTENT OF BLOOD*

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DURING the course of our work upon the experimental production of pulmonary fat embolism,[1] it was found that the tolerance for fat injected intravenously appeared to vary with the animal's nutrition and diet. Therefore it became necessary to control these experiments by determining the lipoid content of the blood before and after the injection of fat. Accepted methods for the determination of blood lipoids, such as the Bang-Bloor chromate titration method or the gasometric method of Backlin, seemed unnecessarily detailed and laborious since we were primarily interested in relative variations of the total lipoid content rather than exact quantitation of the individual lipoid constituents. Our search for a simple procedure disclosed the Hemolipokrit method recently devised by Rückert[2] of Marburg, Germany. With a few modifications, this simple, volumetric micromethod has proved to be ideally suited to our needs, and we feel that it is of such potential value in investigative work upon "fats" that a detailed report of our experience with the method should be placed on record.

Throughout this report the term *lipoid* is used in accordance with the terminology employed by Peters and Van Slyke[3] to denote the fats and fatlike substances in general, while the conjugated lipoids, such as the phosphatids and cerebrosides, are designated by the term *lipides*.

The principle of the Lipokrit method is essentially that of the Babcock method for determining the fat content of milk and cream. The proteins of the blood are changed from the colloidal state to one of molecular dissociation by means of sulphuric acid, permitting complete liberation of the lipoids by hydrolysis and centrifugation. Rückert found the optimum concentration of sulphuric acid for freeing lipoids from blood serum to be 70 per cent by weight when used in the proportion of five parts by volume of acid to one part of serum. He also devised the hemolipokrit pipette which made it possible to measure accurately the minute volume of lipoids derived from small amounts of blood (Fig. 1). The addition of a small amount of amyl alcohol to the sulphuric acid accelerates the separation of the fat droplets and facilitates their coalescence into a measurable column.

In testing the accuracy of this method, Rückert employed aqueous emulsions of the various lipoids and recovered them from the sulphuric acid, amyl alcohol mixture with an average error of only 0.004 gm. per cent. He found that

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neutral fats, fatty acids, cholesterol, and cholesterin esters separated immediately upon centrifugation, while phosphatids, such as lecithin, required an eleven-hour period of hydrolysis for complete liberation of their fatty acids. Mixed aqueous emulsions of equal parts of triolein and lecithin were found to yield an increasing volume of fat and fatty acids with hourly centrifugations until a maximum constant level was reached at the end of eleven hours (Fig. 2). The

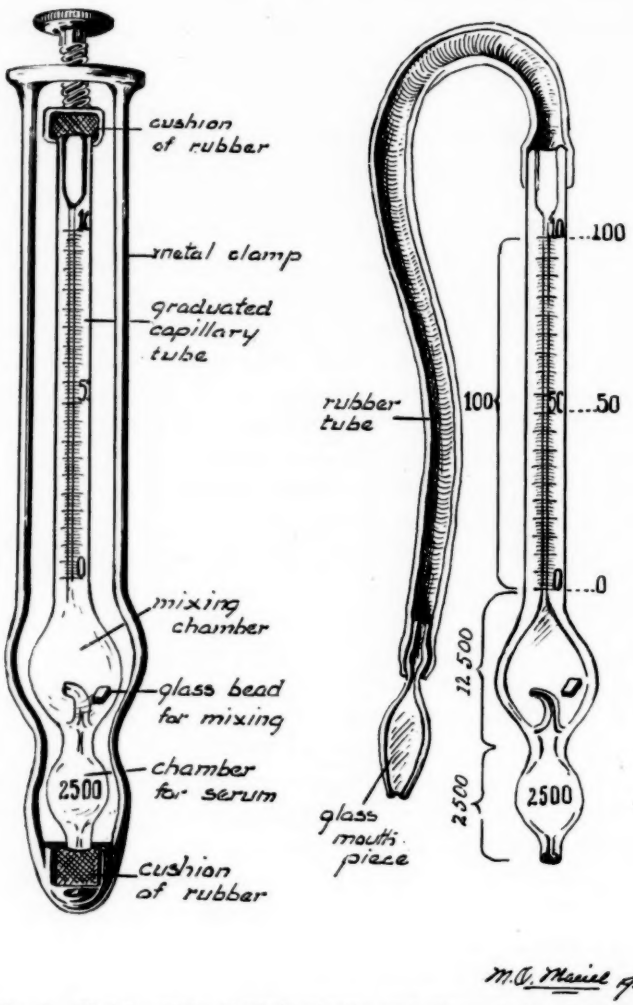

Fig. 1.—Sketch of the lipokrit pipette and clamp (after Rückert).

difference between the initial and final readings, therefore, represented the fatty acids derived from lecithin.

By applying the foregoing observations to the examination of blood serum, Rückert was able to establish a simple lipoid partition by which the neutral fats, free fatty acids, cholesterol and cholesterin esters were obtained immediately upon centrifugation in the first fraction, while the various lipides were represented by their fatty acids in the second fraction obtained after eleven to twenty-hour hours of hydrolysis. In order to compute the volume of lipides

from their fatty acids in the second fraction, it was necessary to multiply the value for this fraction by the arbitrary factor 1.1964 obtained by assuming that lecithin, which bears a volumetric relationship of $\frac{739.4}{618}$ or 1.1964 to its fatty acid constituents, is the only important lipide of blood serum. The *total lipoid content* of a specimen of serum was then determined by adding the observed volumes-percentage of lipoids obtained in the first fraction to the computed volumes-percentage of lipides in the second fraction. Further differentiation of the lipoid partition may ultimately be possible. In serum of known high cholesterol content we have been able to demonstrate, with the polarizing microscope, a definite anisotropic layer at the base of the fat column in the lipokrit stem, but so far we have not succeeded in making consistent measurements of this layer.

At present there is no extensive work on the comparison of the results of the lipokrit method with those of other methods. Rückert,[2] and Leppien[4] have made parallel volumetric and gravimetric determinations of the total lipoid content of serum. In these studies the results obtained by the volumetric method were converted from milliliters-percentage (ml.%) to grams-percentage (gm.%) by (A) applying an arbitrary specific gravity factor (obtained by assuming, as do Bloor[5] and Backlin,[6] that the mixture consists of two parts of fat to one of cholesterol) to the value for the mixed lipoids of the first fraction, and (B) by dividing the value of the lipides of the second fraction by the specific gravity of lecithin. The gravimetric values were obtained by extracting the lipoids with alcohol, ether, and petroleum ether and evaporating the filtrate to constant weight. The eight pairs of parallel determinations made by Rückert showed an average difference between volumetric and gravimetric results of 0.011 gm. per cent, with discrepancies ranging from 0.001 gm. per cent to 0.036 gm. per cent. In all but two, the volumetric value was the higher. In a similar series reported by Leppien the differences ranged from 0.003 gm. per cent to 0.066 gm. per cent, with an average discrepancy of 0.029 gm. per cent. All but three of these pairs showed a higher gravimetric value.

METHOD

*I. Apparatus.**—In devising the hemolipokrit pipette Rückert combined the essential features of the hematocrit tube, the erythrocytometer pipette and the Babcock bottle, adding a small inlet bulb one-fifth the volume of the mixing chamber, for measuring the proper proportion of serum. The serum bulb, the mixing chamber, and the graduated portion of the capillary stem bear a volumetric relationship to one another of 25:125:1. Accurate measurement of the sample of serum is facilitated by the addition of an "automatic" capillary tip which projects into the mixing chamber from the serum bulb (Fig. 1). The capillary stem is so calibrated that each of its 100 divisions represents 0.04 per cent of the capacity of the inlet or serum bulb. The actual volume of this bulb will vary slightly in different pipettes but the average capacity is 0.15 ml.

The hemolipokrit clamp is a great improvement over the ordinary hematocrit clip. The pipette can be held tightly enough in this clamp to permit violent shaking and high-speed

*The lipokrit pipettes are manufactured by Arno Haak, Jena, Germany. They are obtainable in the United States from the Cincinnati Scientific Company, 210 East Second Street, Cincinnati, O.

centrifugation without leakage. The difference in weight between the various pipettes and clamps is negligible thus eliminating the necessity for balancing before centrifugation.

II. Reagents.—(a) Sulphuric Acid: This reagent should be an aqueous solution of sulphuric acid, 70 per cent *by weight* with a specific gravity $\left(\frac{20^\circ\ C.}{4^\circ\ C.}\right)$ of 1.6105. Rückert found that more concentrated solutions oxidize the lipoids to a certain extent, while more dilute solutions are incapable of completely hydrolyzing the coagulated protein. We have confirmed these observations and, in addition, have found that a solution of sulphuric acid of greater specific gravity will give fictitiously high fat readings (detected by control tests with physiologic salt solution) by causing amyl alcohol to separate from the mixture. Since sulphuric acid (c. p.) varies somewhat in concentration, we have found it necessary to determine its specific gravity by means of a Westphal balance prior to making the calculations. It is also advisable to check the specific gravity of the solution which has been prepared to detect any possible error.

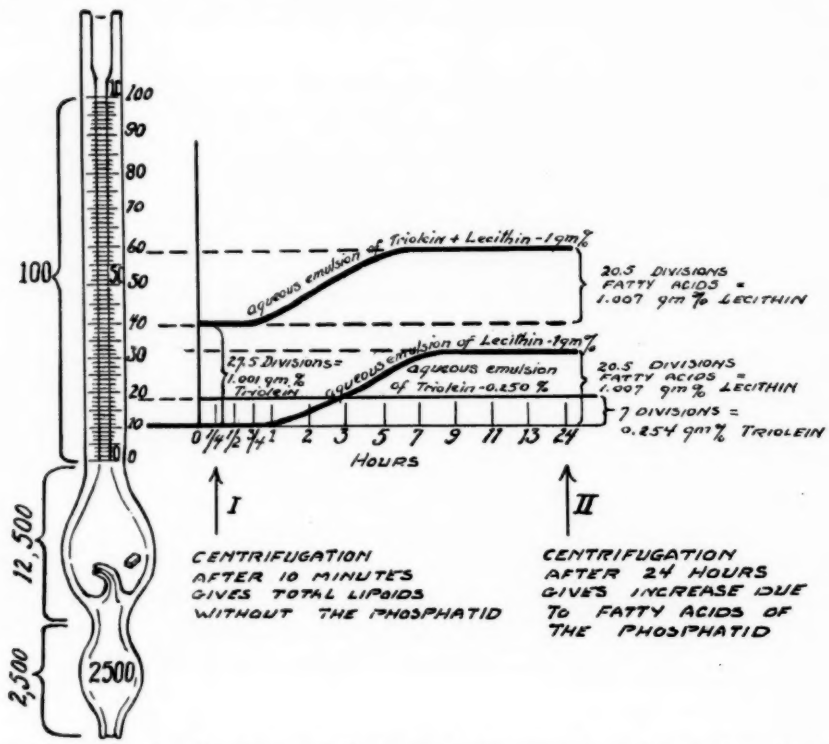
100
12,500
2,500
2500
100
90
80
70
60
50
40
30
20
10
0
Aqueous emulsion of Triolein + Lecithin - 1 gm %
20.5 DIVISIONS FATTY ACIDS = 1.007 gm % LECITHIN
Aqueous emulsion of Lecithin - 1 gm %
27.5 DIVISIONS = 1.001 gm % Triolein
Aqueous emulsion of Triolein - 0.250 %
20.5 DIVISIONS FATTY ACIDS = 1.007 gm % LECITHIN
7 DIVISIONS = 0.254 gm % TRIOLEIN
0 1/4 1/2 3/4 1 2 3 5 7 9 11 13 24
HOURS
I
II
CENTRIFUGATION AFTER 10 MINUTES GIVES TOTAL LIPOIDS WITHOUT THE PHOSPHATID
CENTRIFUGATION AFTER 24 HOURS GIVES INCREASE DUE TO FATTY ACIDS OF THE PHOSPHATID

Fig. 2.—Sketch showing the time hydrolysis curve for aqueous emulsions of various lipoids (after Rückert).

(b) Amyl Alcohol: Chemically pure iso-amylic alcohol with a boiling point of 128° to 130° C., and a specific gravity $\left(\frac{15^\circ\ C.}{4^\circ\ C.}\right)$ of 0.850 has been found more satisfactory for this method than the fusel oil ordinarily employed in milk analysis.

(c) Contrast Stains: In order to facilitate measurement of the lipoid column in the capillary stem of the lipokrit, Rückert stained the sulphuric acid with methylene blue and made the amyl alcohol a vehicle for a spirit soluble anilin yellow dye which is taken up by the fatty acid radicle. The clear, colorless fluid column in which the fatty layer is distinguishable only by the light reflex of the interface, is differentiated by this means into two sharply contrasting components—an opaque dark blue green column rising out of the mixing chamber and a supernatant pale yellow lipoid layer. The optimum concentration of these dyes in their respective solvents was found to be as follows: methylene blue (medicinal) 0.08 gm. per 100 ml. of the 70 per cent sulphuric acid solution, and anilin yellow

0.15 gm. per 100 ml. of amyl alcohol. We have found that a saturated solution of scharlach R in amyl alcohol is a much more effective stain for the lipoid layer than any anilin yellow dye which we have been able to obtain in this country. The bright red color imparted to the lipoids affords a much more striking contrast with the blue green color in the stem of the lipokrit pipette.

III. Technic.—(a) Preparation of the Blood Sample: The blood is taken from an artery or vein with small glass syringes which have been cleaned in sulphuric acid—potassium dichromate solution and dried with alcohol and ether, using rustless steel needles rendered fat-free by acetone and sterilized in the autoclave. In order to prevent hemolysis the needles and syringes must be absolutely dry. Two milliliters of blood is ample for duplicate de-

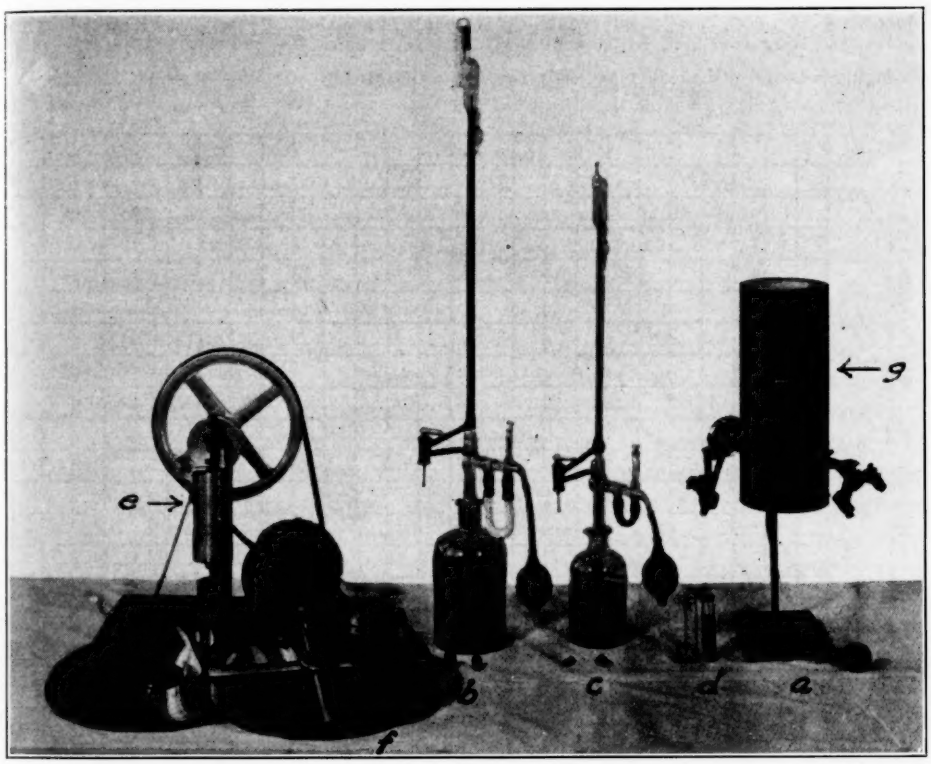

Fig. 3.—Photograph of the accessory apparatus. *a*, Capillary pipette for separating the serum. *b*, Automatic burette bottle for sulphuric acid, methylene blue solution. *c*, Automatic microburette bottle for amyl alcohol scharlach R solution. *d*, Glass stoppered weighing bottles for mixing the special reagent. *e*, Motor-driven shaking machine for the lipokrit pipettes. *f*, Special duralumin centrifuge head. *g*, Heat insulated slit lamp with magnifying glass and microburner attached.

terminations. The specimens are transferred to chemically clean, fat-free culture tubes 5 inches in length, and then centrifuged as soon as the clot has formed. We have found that centrifugation at approximately 1900 r.p.m. for five minutes in a No. 1C International centrifuge with head No. 233 fitted with the usual small cups gives the maximum yield of serum with a minimum demulsification of the fats. The supernatant serum is then transferred to another tube by means of a special large capillary pipette drawn from a 6-inch hard glass culture tube and fitted with an "asepto" rubber bulb (Fig. 3, *a*).

(b) Preparation of the Reagent Mixture: The sulphuric acid-amyl alcohol mixture is prepared by adding 1 volume of the amyl alcohol-scharlach R solution to 12.5 volumes of the sulphuric acid-methylene blue solution. Rückert found that when smaller proportions of alcohol are used the fat droplets tend to remain discrete and fail to coalesce properly upon

centrifugation, while larger proportions of amyl alcohol interfere with the hydrolysis of the proteins. Furthermore, the mixture should be *freshly* prepared for each set of determinations because amylene is formed on standing and will rise with the lipoids upon centrifugation and give fictitiously high fat readings. We have confirmed these findings to the extent of observing somewhat higher readings with the use of sulphuric acid-amyl alcohol mixtures which were twenty-four to seventy-two hours old. We have found it most convenient to keep these solutions in "all automatic" burette bottles (Fig. 3, *b* and *c*), using a microburette for the amyl alcohol, and protecting the sulphuric acid solution from atmospheric moisture by "trapping" the vents with calcium chloride tubes and stoppering them when not in use. With this equipment small amounts of reagents may be measured out and freshly mixed for each set of determinations with a minimum of waste and exposure, and a maximum of speed and accuracy. Approximately 0.75 ml. of the mixture is needed for each determination, consequently a series of 12 determinations would require 10 ml. of the sulphuric acid-methylene blue solution and 0.8 ml. of the amyl alcohol-scharlach R solution. These fluids should be thoroughly mixed by shaking vigorously for five minutes and warming to about 50° C. For

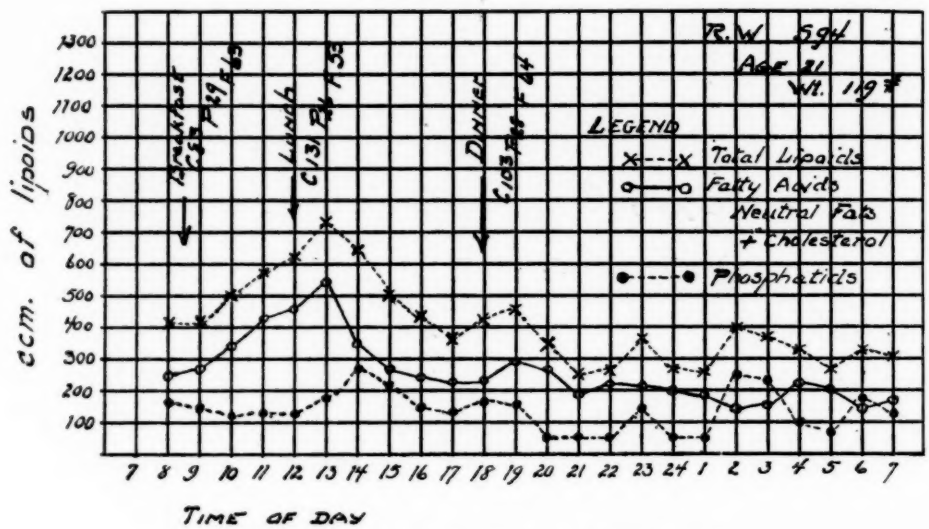

Fig. 4.—Alimentary lipemia curve of a normal adult.

this purpose narrow 4-inch glass-stoppered weighing bottles (Fig. 3, *d*) have proved most convenient. After mixing, the reagent should be allowed to stand for about twenty minutes or until it has cooled to room temperature and is free from bubbles. We have found that the premature use of this mixture causes a collection of foam in the lipokrit stem which interferes with the coalescence of the fat droplets. *The reagent should, therefore, be prepared about thirty minutes before it is to be used.*

(c) Filling the Pipettes: The serum should be agitated by gentle rotation of the tube to insure homogeneous distribution of the lipoids which sometimes come to the top of the serum during centrifugation. Violent shaking should be avoided because of the inconvenience of the resulting bubbles. The lipokrit pipette is then introduced into the tube and the serum is slowly drawn up into the measuring bulb until the automatic capillary tip is filled. The pipette is withdrawn, held horizontally while the end is wiped dry and then dipped into the reagent mixture. This is sucked up *quickly* through the inlet bulb to prevent coagulation of the serum in the automatic tip, and then drawn *very slowly* into the mixing chamber. During this process the pipette should be held vertically and twirled rapidly to and fro between the thumb and forefinger as the fluid rises. At least one minute should be consumed in filling this chamber, for if the process is hurried, dissociation of the coagulated protein will be sufficiently delayed to retard the liberation of the lipoids and lead to low readings

after the initial centrifugation. The reagent should be allowed to rise but halfway up the capillary stem, and when it has been adjusted at the 50 mark, the pipette should be withdrawn to the horizontal position, wiped dry, and fitted tightly into the lipokrit clamp. The apparatus is then shaken vigorously in a motor-driven device (Fig. 3, *e*) for five minutes. If the time intervals for filling and shaking have been properly observed, the fluid column will be found to have contracted with cooling to the level of the 10 or 15 mark at the base of the capillary stem, and this incompletely hydrolyzed portion may be allowed to run back into the mixing chamber without fear of losing any lipoid material at the inlet. Filling of the pipette is then completed by again drawing up reagent until the column reaches the 100 mark at the top of the stem. The objections to filling the pipette in one stage are, first, incomplete hydrolysis of the material in the stem, and second, contraction of the fluid column into the mixing chamber upon cooling. If the pipettes are filled properly in two stages, the fatty layer will always be found within the limits of the graduated portion of the capillary stem.

(d) Centrifugation: In his original publication of this method, Rückert specified centrifugation at a speed of 2,000 r.p.m. for ten minutes, but later in a personal communication to us he stated that he had found it necessary to increase the speed of centrifugation to 4,000

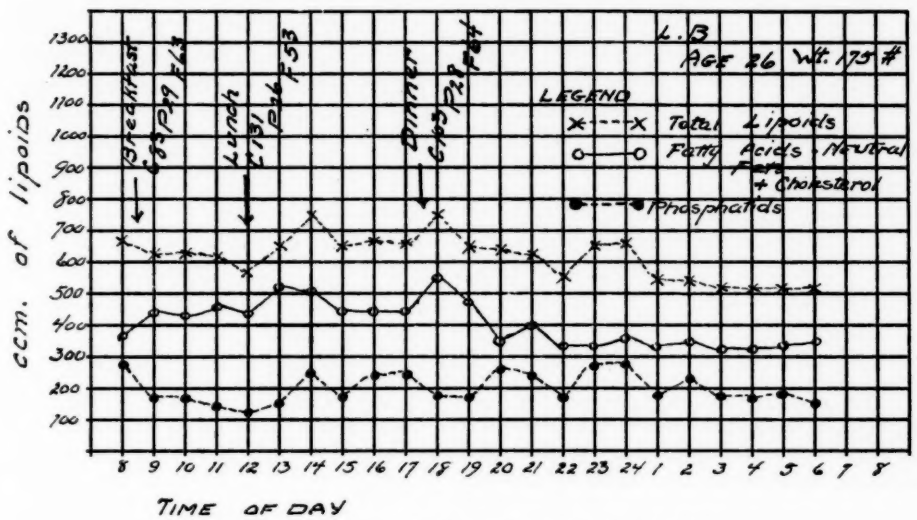

Fig. 5.—Alimentary lipemia curve of an obese adult.

r.p.m. in order to obtain the most constant and accurate results. We have found that the speed of centrifugation (the radius remaining constant) must be governed by the time hydrolysis curve of the lipides in relation to the total (elapsed) time necessary for filling, shaking, and centrifuging a given number of pipettes. By repeated determinations at various speeds on the same specimen of serum, with centrifugation for periods of five minutes at five-minute intervals, we have learned that the lipoids of the first fraction are usually liberated completely in from ten to thirty minutes after filling the pipettes, depending upon the degree of centrifugal force applied. We have also found that the hydrolysis of the lipides commences after thirty minutes have elapsed regardless of the speed and duration of centrifugation. *It seems to be essential, therefore, that readings for the first fraction be completed within thirty minutes after filling the pipettes.* If twenty minutes were required to fill and shake 4 pipettes then they should be centrifuged with sufficient force to liberate the entire first fraction in the remaining ten minutes, while if only 2 determinations were to be made, requiring but ten minutes for filling and shaking, there would be twenty minutes left for centrifugation and the separation could be accomplished at a slower speed with a centrifugal force which would be inadequate for a shorter interval. The "sedimentation-time" for lipoid emulsions may be said to be approximately inversely proportional to the relative centrifugal force

(R. C. F.). Example: Using an International centrifuge No. 1C with head No. 233 and 2 small carriers (rotating radius of the center of gravity of the fluid mass being 15 cm.) at the maximum obtainable speed of 1,900 r.p.m., we were able to apply a centrifugal force of only 589,235 dynes/gm. (R. C. F. 601 gm./gm.), and complete liberation of the first lipoid fraction required a twenty-minute period of centrifugation; while with the same centrifuge but a special duralumin head (radius 11.25 cm.) designed to reduce "windage," a speed of 3,500 r.p.m. was obtained, and the resulting centrifugal force of 1,502,156 dynes/gm. (R. C. F 1,533 gm./gm.) was sufficient to separate these lipoids in about eight minutes. Theoretically this work could be accomplished in approximately one minute by an R. C. F. of 12,000 gm./gm. From these observations the following proportion may be stated, by means of which it is possible to estimate the *number of minutes of centrifugation* (T) required for any given combination of *speed* (N in r.p.m.) and *radius* (R in cm.), i.e.,

$$T : 1 :: 12{,}000 : 0.0000111N^2R$$

$$T = \frac{1{,}081}{\left(\frac{N}{1{,}000}\right)^2 R}$$

If T *plus* the time required for filling and shaking the desired number of pipettes is found to be thirty minutes or more, a greater centrifugal force will be needed. Although most of the determinations which we present in Tables I and II were made with an R. C. F. of 600 gm./gm. for fifteen minutes and a total (elapsed) time of twenty minutes for separating the first fraction, we are now convinced that in order to obtain comparable and uniform results *the initial centrifugation should be made consistently with an R. C. F. of 1,200 gm./gm., for a period of ten minutes and that the total elapsed time (from filling to reading) should be uniformly limited to twenty-five minutes.* The specified centrifugal force may be obtained with the following equipment:

MAKE OF CENTRIFUGE	MODEL	TYPE OF HEAD	SPEED
International	1C	Special (Fig. 3, *f*)	3,000 r.p.m.
International	1SB	Nos. 210; 220; 225	2,900 r.p.m.
International	2	Nos. 251; 240; 241	2,700 r.p.m.

(e) The First Fraction: Readings. After centrifugation the fluid column will usually be found to have contracted to the 70 or 80 mark on the lipokrit stem, and the coalesced, liberated lipoids, stained a bright scarlet, will be layered over the dark blue green reagent mixture at about this level. The length of the lipoid column should be read promptly with the aid of a magnifying glass and a bright light. Because of the thermometer-like behavior of the sulphuric acid, a cool source of light is desirable, and we have found that readings are facilitated by the use of a heat-insulated slit-lamp (Fig. 3, *g*). Frequently a portion of the lipoids solidifies on cooling, and in order to obtain a clear-cut interface it is necessary to warm the portion of the lipokrit stem occupied by the fatty layer in the flame of a microburner. There is no appreciable expansion of the column of fat on heating. In cold weather it is advisable to do this routinely as quickly as possible, because slight warming of the bulbs from contact with the hands or lamp will make the fluid rise and leave solidified lipoid particles behind thereby necessitating recentrifugation. Each division of the lipokrit scale is equivalent to 0.04 per cent of the volume of the serum taken into the pipette. It is possible to read accurately to one-tenth of one division. For example, a fatty layer occupying 9.6 divisions would represent a content of 0.384 ml. of neutral fat, free fatty acids, cholesterol and cholesterin esters per 100 ml. of serum. In view of the differences in specific gravity and variations in the relative proportions of these lipoids, we prefer to report the value for the first fraction in terms of the observed relative volume rather than attempt to transpose it into gravimetric units.

(f) The Second Fraction: Time hydrolysis curve of the lipides. After taking the initial readings, Rückert allowed the pipettes to stand for twenty-four hours in an incubator at 37° C., in order to obtain complete hydrolysis of the lipides. We have learned, however, from

parallel determinations at 37° C., and at room temperature (22° C. to 27° C.), that the rate and completeness of hydrolysis are not influenced appreciably by temperature within these limits. We have also observed that somewhat lower readings are frequently obtained after incubation at 37° C. due to the loss of small amounts of lipoid material which adhere to the glass above the capillary bore when the fatty layer is carried to the top of the stem on

TABLE I

VOLUME OF LIPOIDS IN RELATION TO THE PERIOD OF HYDROLYSIS

	DONORS OF SERUM PERIOD OF FASTING, FOURTEEN HOURS	NO. OF LIPOKRIT	VOLUME OF THE LIPOIDS (RECORDED IN DIVISIONS OF LIPOKRIT SCALE)				
			30 MINUTES	4 HOURS	8 HOURS	12 HOURS	24 HOURS
1	White male, aged 30, Ht. 65″,	211	5	10	11	11	11.5
	Wt. 143 pounds. Interne.	212	5	10	11	11	11.5
	Hospital diet	213	5	10	11	11	11.5
2	White male, aged 26, Ht. 65″,	237	6	10	12	12	12
	Wt. 145 pounds. Interne.	239	6	10	11	12	12
	Hospital diet	240	6	10	11	12	12
3	White male, aged 28, Ht. 70″,	231	9	14	14	15	15
	Wt. 158 pounds. Interne.	232	9	14	14	15	15
	Hospital diet	233	9	13.5	14	14	15
4	White male, aged 29, Ht. 72″,	231	9	13	14	14	14
	Wt. 175 pounds. Interne.	232	9	14	15	15	15
	Hospital diet	233	9	13	14	14	14
5	White male, aged 25, Ht. 72″,	234	7	10.5	12	12	12
	Wt. 175 pounds. Interne.	235	7	11	12	12	12
	Hospital diet	236	7	10.5	12	12	12
6	White female, aged 47, Ht.	235	7	13	14	14	14.5
	64″, Wt. 250 pounds. Hy-	236	7	13	14	14	14
	pertension. 1,200 cal. diet	237	7	12	13	14	14
7	White female, aged 48, Ht.	234	10	12	15	15.5	15.8
	65″, Wt. 225 pounds. Obst.	235	10	13	15	15.5	16
	jaundice. Hospital diet	236	10	13	14.5	15.3	16
8	White female, aged 24, Ht.	231	20	26	26	27	27
	65″, Wt. 140 pounds. Preg-	232	21	26	26	27.5	27.5
	nancy, term. Hospital diet	233	20	26	26	27	27
9	White female, aged 24, Ht.	211	10	10	11	12.8	12.9
	55″, Wt. 166 pounds. Tox-	212	9	10	12	13	13
	emia, preg. Nephritic diet	213	9	10	13	13	13
10	White male, aged 52, Ht. 65″,	222	2.7	13	--	17	17.5
	Wt. 127 pounds. Catar.	223	2.4	12.2	--	16	17
	jaundice. Fat-free diet	224	2.5	12	--	17.5	17.5

TABLE II

DIVISIONS → / ↓		UNITS									
		0	1	2	3	4	↑ 5	6	7	8	9
TENS	0	0	0.048	0.096	0.144	0.191	0.239	0.287	0.335	0.383	0.431
	1	0.478	0.526	0.574	0.622	0.670	0.718	0.765	0.831	0.861	0.909
	2	0.957	1.005	1.053	1.100	1.148	1.196	1.244	1.292	1.340	1.387
	3	1.435	1.483	1.531	1.579	1.627	1.674	1.722	1.770	1.818	1.866
	4	1.914	1.961	2.009	2.057	2.105	2.153	2.201	2.248	2.296	2.344
	5	2.392	2.440	2.488	2.536	2.583	2.631	2.679	2.727	2.775	2.823

warming. Consequently, we prefer to carry out this process at room temperature. Furthermore, we believe that there is not sufficient difference between twelve-hour and twenty-four-hour readings to justify depriving the laboratory of the use of the lipokrit pipettes for more than twelve hours. Evidence for this opinion is presented in Table I of 10 determinations in triplicate (Table I). After the twelve-hour period of hydrolysis, the fatty acids derived from lipides are added to the lipoids of the first fraction by a second period of centrifugation which should be identical with the first.

IV. Calculations.—The value for the reading obtained after the twelve-hour period of hydrolysis does not represent the total lipoid content of the

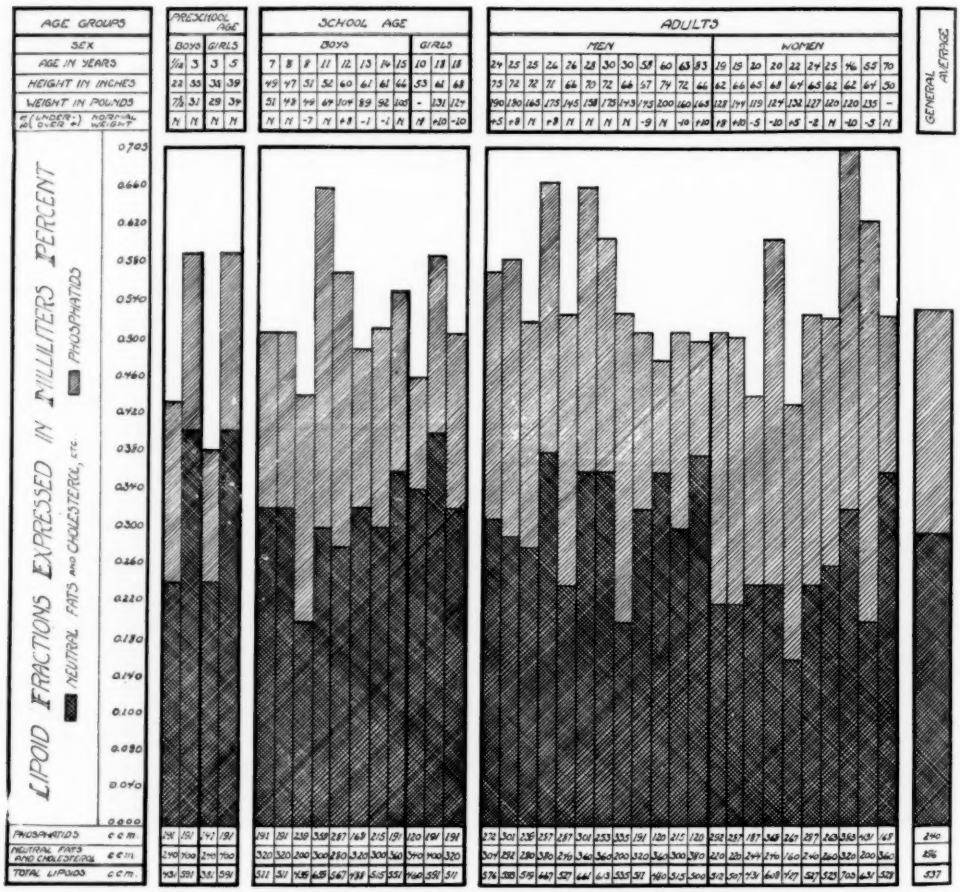

Fig. 6.—Chart showing the lipoid content of the blood of 37 normal patients as determined by the lipokrit method.

serum because the increment, or the difference between the first and second readings, is due to fatty acids derived from the lipides and not due to the lipides themselves. The apparent volume of the lipides must be computed from the observed volume of their constituent fatty acids by multiplying the number of scale divisions of the increment by 0.04 and then by the factor 1.1964, or this may be done more simply in one step by multiplying by the factor 0.048. *The total lipoid content of the serum may then be determined by adding this value for the apparent volume of the lipides to the observed volume of the lipoids in*

the first fraction. These calculations are greatly facilitated by the use of Table II prepared by Rückert, in which the ml. per cent of lipides (phosphatids), computed from their fatty acid components, is given for any number of divisions of the lipokrit scale from 1 to 59.

V. Results.—Fig. 6 shows the volume of lipoids in the blood serum, after fourteen hours of fasting, as determined by the lipokrit method in a series of 37 normal patients.

Since only a small quantity of blood is needed for each set of determinations made by this method, the rate and degree of absorption of fat from the alimentary canal can be studied easily and without injury to the patient. Several normal adults volunteered for such studies. All of these patients were given the same diet of known composition. The lipoids of the blood were determined at hourly intervals for twenty-four hours. The results of two of these series of determinations are shown in the accompanying curves (Figs. 4 and 5). A more detailed report of similar studies will be presented at some later time.

SUMMARY

The lipokrit method is a simple volumetric micromethod for the determination of the lipoid content of blood serum.

Slight alterations of the original method of Rückert with certain refinements of technic have made it possible for us to obtain consistently uniform results.

The lipoid content of the blood serum, after fasting for about fourteen hours, appears to be fairly constant regardless of the age or sex of the individual.

Two curves showing the typical increase in the lipoids of the blood which occurs several hours after each meal are presented.

NOTE: The authors wish to express their appreciation to Dr. Glenn E. Cullen, Director of Laboratories of the Children's Hospital Research Foundation, for his kind cooperation and many valuable suggestions during the course of this work.

REFERENCES

1. Herrmann, L. G.: Effect of Dextrose-Alcohol Mixture upon Pulmonary Fat Embolism, Proc. Soc. Exper. Biol. & Med. **30**: 558, 1933.
2. Rückert, W.: Eine einfache volumetrische Mikrobestimmung des Blutfettes (Lipokritverfahren), Klin. Wchnschr. **10**: 1853, 1931.
3. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry Methods **2**: Baltimore, 1931, Williams and Wilkins Company, pp. 218.
4. Leppien, R.: Über den Blutfettspiegel und den zeitlichen Verlauf seiner Veränderung unter dem Einfluss eines Ölfrühstücks beim gesunden und kranken Menschen, Ztschr. f. ges. Exper. Med. **81**: 728, 1932.
5. Bloor, W. R.: The Determination of Small Amounts of Lipid in Blood Plasma, J. Biol. Chem. **77**: 53, 1928.
6. Backlin, E.: Eine Mikromethode zur quantitativen Bestimmung der Lipoide, Biochem. Ztschr. **217**: 483, 1930.

THE MODIFIED GRAM STAIN OF MUCH*

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BMÖLLERS, in the *Handbuch der pathogenen Mikroorganismen* (1930), states that "in the various forms of human tuberculosis one finds only the acid-fast form of the tubercle bacillus, or one attributes only to this form a diagnostic value." In a previous paper[1] by me it was seen that the classical tubercle bacillus has no genuine Gram property as long as the organism is acid-fast; moreover, that it cannot have this property because of the presence of the acid-fast lipoids. It was found further that when the tubercle bacillus was completely deprived of its acid-fast material, it became gram-negative. H. Much,[2] however, claimed to have found, with the aid of a modified Gram staining technic, a pathogenic granular nonacid-fast form of the tuberculosis virus, a form hitherto unknown. According to his findings this organism, when stained by his method, is found to consist either of a very short, beaded fragmented rod or of isolated granules, or heaps of granules, all of which are nonacid-fast and gram-positive.

When one studies the Gram technic of Much, it will be seen that the latter employs two carbol dyes such as are used in acid-fast staining, viz., carbol-fuchsin 3 parts, carbol methyl violet 1 part. Moreover, Much's technic requires prolonged cold staining up to forty-eight hours. In order to give the method a semblance of the Gram stain, the organisms are then exposed to hot iodine solution for five minutes. This is followed successively with 5 per cent nitric acid for one minute, 3 per cent hydrochloric acid for ten seconds, and finally with equal parts of 95 per cent alcohol and acetone. These latter are acid alcohol acetone decolorizers that are even more powerful in their action than those applied in the classical Ziehl-Neelsen acid-fast method, in which only 3 per cent hydrochloric acid in 70 per cent alcohol is employed. Consequently, any microorganisms retaining the Much dyes after being treated with these decolorizers must be considered not merely alcohol-fast as required of gram-positive organisms but acid-fast as well. Hence, this led me to conclude that the so-called modified Gram stain of Much is really an acid-fast staining technic, and that the Much granules are in reality acid-fast bodies. In order to observe the action of Much's staining method on acid-fast tubercle bacilli, some experimental work was carried out in which this staining technic was analyzed into its separate procedures.

Experiment 1.—Purpose: To determine the action of the Much's modified Gram staining technic on untreated acid-fast tubercle bacilli. Exposure time, forty-eight hours. The tubercle bacilli stained an intense purplish blue especially the granules which stand out like cocci either in rows or in clusters. One could scarcely note any difference between these and Much's granules as found in the pus from cold abscesses. None of the bacilli stained red, the purplish blue color predominated (Fig. 1).

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Experiment 2.—Purpose: To determine the effect upon color retention of untreated tubercle bacilli stained with aniline gentian-violet as employed in the standard Gram stain, of the decolorizing agents employed in the Much staining technic.

A. Tubercle bacilli were stained on slides at room temperature for one minute with aniline gentian-violet. Gram's iodine was applied for five minutes hot as in Much's technic. Decolorization with 5 per cent nitric acid for one minute, 3 per cent hydrochloric acid for ten seconds, acetone alcohol until color ceases to come off.

Results: Organisms were well stained. They retained their color after use of Much's decolorizers.

B. Tubercle bacilli were treated as under A, with one-minute exposure to cold Gram's iodine solution.

Result: Organisms were equally well stained as in A.

C. Tubercle bacilli were treated as under A, but without iodine.

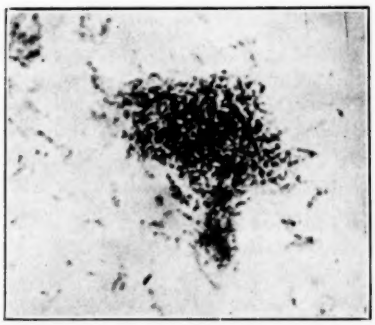

Fig. 1.—Tubercle bacilli of the beaded type which have been stained according to Much's technic, resembling Much's "granules." Note intergranular bridges, one of the requisites of true "Much granules."

Result: The organisms retained stain in spite of lack of application of iodine when the Much acid acetone alcohol decolorizing solutions were employed.

Experiment 3.—In this experiment the Much modified Gram stain was analyzed by staining untreated intact pulverized living tubercle bacilli with the separate components of this technic in order to ascertain whether all the steps of the Much technic tend to increase its efficiency in tinctorially demonstrating the tubercle bacillus. The results of the experiment are recorded in Table I.

Comment on Experiment 3: It will be seen in B of Table I that when the entire system of decolorizers employed in Much's staining technic is employed, the intensity and the quality of red of the carbolfuchsin are greatly altered so that the tubercle bacilli are scarcely recognizable. On the other hand as seen in A of the table the methyl violet is but little altered in these respects by the same decolorizers.

From the above staining analysis, by the process of elimination it appears that the principle offender in causing alteration of the color quality and intensity of the carbolfuchsin is the nitric acid. This acid changed the brilliant red of the carbolfuchsin either to a pale lavender or entirely decolorized the tubercle bacillus of this stain whether iodine was used or not. The action of nitric acid on methyl violet, however, is not so marked. Hence, the carbolfuchsin adds practically nothing to the end-staining result since the nitric acid nearly decolorizes this stain when applied to the tubercle bacillus in the course of the Much technic.

Moreover, it will be seen that the tubercle bacillus stains as well and better without, as with, iodine. When iodine was applied after the carbol methyl violet, the former produced an annoying granular precipitate which obscured the tubercle bacilli and simulated the granules of the latter. It causes a change in color quality and staining intensity of the carbolfuchsin.

It is consequently evident that in employing the Much staining technic

TABLE I

	TUBERCLE BACILLI STAINED WITH:			
	A. CAPBOL METHYL VIOLET		B. CARBOLFUCHSIN	
DECOLORIZER	WITH IODINE	WITHOUT IODINE	WITH IODINE	WITHOUT IODINE
1. HNO_3, HCl, Acetone alcohol	Intensely stained	Intensely stained	Practically not stained	Poorly stained. Nearly decolorized
2. HNO_3, Acetone alcohol	Well stained	More intensely stained than with iodine	Practically not stained	Practically not stained
3. HNO_3, HCl	Stain precipitated in small granules, obscuring tubercle bacilli	No precipitate. Bacilli well stained	Very poorly stained, a lavender color	Very poorly stained, a lavender color
4. HNO_3	So much granular precipitate as to obscure bacilli	No precipitate. Bacilli well stained	None bright red; some pink	Not bright red. Lavender. Most organisms not stained or poorly so
5. HCl, Acetone alcohol	No precipitate. Not as intensely stained as (1) above	No precipitate. Bacilii well stained	Practically not stained	Well stained. Bright red
6. HCl	Much granular precipitate obscuring bacilli	No precipitate. Bacilli well stained	Not distinctly stained	Stained bright red
7. Acetone alcohol	No precipitate. Bacilli well stained	Slight granular precipitate. Bacilli not obscured by precipitate. Latter well stained	None bright red. Poorly stained	Well stained, red
8. Acetone	Granular precipitate. Bacilli well stained	No precipitate. Bacilli well stained	Lavender tubercle bacilli. None red. Faintly stained	Stained red
9. Alcohol	Much granular precipitate. Bacilli well stained	No precipitate. Bacilli well stained	No stained tubercle bacilli at all	Well stained, red

we are not dealing even with a modified Gram stain but with an acid-fast stain for the following reasons:

a. The component dyes of the Much stain are typical acid-fast stains.

b. The Gram's iodine adds nothing to the value of the Much staining technic since typical tubercle bacilli will stain as well without iodine as with it, in fact, somewhat better without it (see Table I).

c. Tubercle bacilli stained with Much's stain will retain the latter (whether treated with iodine or not) not only after decolorization with alco-

hol, as is to be expected of a gram-positive organism, but also after decolorization with nitric and hydrochloric acids, and acetone alcohol. Hence the stain is an alcohol acid-fast stain and not a Gram stain.

d. In these experiments the tubercle bacillus has been used to determine the acid-fast property of the Much stain, since this organism is generally considered the most acid-fast one of that group. If, then, it be correct, as asserted by Much, that the so-called Much "granules" are nonacid-fast gram-positive bodies representing an atypical form of the tuberculosis virus, he should have employed a method of staining other than that devised by him, since his is an acid-fast stain and the bodies stained therewith are, consequently, acid-fast "granules" (they are not always granules, but frequently show connecting intergranular bridges or bacillary structure), resembling, in reality, the granular forms of the typical tubercle bacillus.[3]

Experiment 4.—The purpose of this experiment was to ascertain whether the method of concentrating tubercle bacilli affects unfavorably the staining of these organisms by the Much technic. In this method of concentrating tubercle bacilli (and "Much granules") from sputum and pus from cold abscesses, strong alkalis (0.6 per cent sodium carbonate and antiformin which contains sodium hydroxide) are applied for forty-eight hours in order to liquefy the tenacious sputum. The organisms so liberated are then concentrated by centrifugalization and applied in the alkaline sediment to slides, dried and stained.

With this in view pulverized intact acid-fast tubercle bacilli were treated with the above concentrating solutions, centrifuged after forty-eight hours and half of them applied to slides without being washed free from alkalis; the other half was repeatedly washed in distilled water until neutral to litmus and then applied to slides and dried. The unwashed bacilli were then stained as follows:

1. With Much's carbolfuchsin and carbol methyl violet for forty-eight hours, hot Gram's iodine five minutes, decolorized with nitric acid, hydrochloric acid, acetone alcohol.

Results: The tubercle bacilli were stained indistinctly a slate blue color.

2. With carbolfuchsin forty-eight hours, hot Gram's iodine five minutes, decolorized with nitric acid, hydrochloric acid, acetone alcohol.

Result: Tubercle bacilli were stained, but not distinctly, a pale lavender instead of a bright red.

3. With cold carbol methyl violet forty-eight hours, hot Gram's iodine five minutes, decolorized with nitric and hydrochloric acids and acetone alcohol.

Result: Tubercle bacilli stained a dark gray-blue, with the granules well stained, the intergranular substance often was not stained; if stained at all, the latter took the dye faintly as a rule. These tubercle bacilli appear very much like the typical Much "granules."

4. With carbolfuchsin, hot, five minutes; decolorized with acid-alcohol, counterstained with methylene blue.

Result: The tubercle bacilli were stained more distinctly than under (2) but not a brilliant red.

Comment: From the first part of Experiment 4 it will be seen that the alkalis employed to concentrate the tubercle bacilli in sputum or pus from cold abscesses, previous to the application of the Much "granule" staining technic, are distinctly detrimental to the staining of tubercle bacilli with Much's carbolfuchsin and carbol gentian violet both as to intensity and color quality. The tubercle bacilli treated with these alkalis do not stain typically bright red with carbolfuchsin. Only carbol methyl violet, after application of these alkalis, colors the tubercle bacillus with moderate intensity.

The washed bacilli were stained as follows:

1. With hot carbolfuchsin five minutes (as in 4 above), hot iodine solution five minutes and Ziehl-Neelsen acid alcohol decolorizer.

Result: The tubercle bacilli were stained reddish, but not a bright red.

2. With the same as in (1) without the iodine.

Result: These tubercle bacilli stained a brilliant red.

Comment: The second part of Experiment 4 demonstrates the following:

a. That if the alkalis used in concentrating tubercle bacilli are removed by washing from the material to be stained, they will not interfere with the staining property of the carbolfuchsin of the Ziehl-Neelsen stain, but if not properly removed they may render the organisms unrecognizable or difficult to find due to poor staining. This may account for some of the failures to detect tubercle bacilli when the concentration method is employed. This method would not, however, interfere seriously with the discovery of the organisms if stained with the intensely blue staining carbol methyl violet of the Much method. This may consequently account for the greater efficiency of the Much staining method in detecting rare tubercle bacilli after application of the above alkaline concentration method.

b. The addition of iodine in the Much technic alters to a considerable extent the bright red color of the carbolfuchsin but not greatly the color quality of the carbol methyl violet.

Experiment 6.—A freshly isolated culture of virulent streptococci was stained according to Much's technic in order to ascertain the effect of this stain upon a typical Gram positive nonacid-fast organism.

Result: Streptococci were completely decolorized by the acid acetone alcohol decolorizers of the Much technic. Only occasional faint outlines of organisms were visible.

Comment: The Much stain is not a Gram stain but acts as an acid-fast stain when applied to known gram-positive organisms.

DISCUSSION

Much[2] maintained that he had discovered a new form of nonacid-fast tuberculosis virus which he claims to have demonstrated by a staining technic devised by him and designated a modified Gram stain. This virus so stained appeared either as purple isolated granules or as heaps of granules, or as beaded, bandlike structures. Unless these granules were connected by a faint bluish or reddish band, it was said, they should not be considered to be true Much granules.[3] This claim of Much to the discovery of an atypical tuberculosis virus has been supported by some and called into question by many, so that B. Möllers[4] in the *Handbuch der pathogenen Mikroorganismen* states recently that the question is still unsettled. Much, however, in his autobiography recently states that his discovery of the virus now is an established fact of science. Although this assertion may well be questioned, the true nature of the "granules" has not been gone into in this paper. In the work here presented, merely the staining method of Much has been called into question.

First, it is claimed by Much that his staining method is a modified Gram staining technic. The work recorded in this article has shown that this claim of Much is incorrect. That first of all his staining technic is not a modified

Gram stain; and second, that it is an acid-fast and not a nonacid-fast stain. In order to prove the former of these contentions gram-positive organisms were employed. These were shown by Much's method to lose their original stains (carbol genitan violet and carbolfuchsin) upon application of his decolorizers following application of hot Gram's iodine solution. According to Much's method then, these gram-positive organisms would have to be considered gram-negative. This would prove incompatible with our conceptions of a Gram stain. The evident reason for the decolorization of these gram-positive organisms is the presence in the Much decolorizers of two acids, besides alcohol and acetone. For a microorganism to be considered gram-positive it must resist alcohol decolorization after application of iodine solution. If a microorganism resists decolorization with acid, even though iodine solution has been applied, it is an acid-fast organism. Such is the case with the Much technic. It is an acid-fast technic and merely the inclusion by it of Gram's iodine solution which adds nothing to its efficacy does not make it a Gram stain. In order further to prove this assertion acid-fast tubercle bacilli were stained with the Much technic and they retained the Much stain after the application of HCl and HNO_3 decolorizers.

Moreover, it was seen in a previous article[1] that the tubercle bacillus in its acid-fast pathogenic state has no true Gram property, because it requires no iodine to enable it to retain its original stain and is not only alcohol but also acid-fast. That, consequently, its acid-fast property precluded the possibility of its possessing genuine gram-positivity. Hence the retention on part of the tubercle bacillus of the Much stain indicated not gram-positivity but acid-fastness.

Thus it will be seen from the experiments in this paper that the Much stain is not a Gram stain, but is actually a powerful acid-fast stain (carbolfuchsin and carbol methyl violet) that is applied over a prolonged period of time (forty-eight hours), the decolorizers are two strong mineral acids (HCl and HNO_3), followed by acetone and alcohol. The iodine was found in the above experiments to be not only unnecessary but detrimental in the retention of the original stain. It was, moreover, found that when the Much staining technic was applied to a pure culture of living beaded acid-fast tubercle bacilli on slides, the latter presented the characteristics of Much "granules" (see Fig. 1). Consequently, it appears from this work that the bodies called Much granules are really acid-fast entities, and not nonacid-fast gram-positive bodies as claimed. No other known nonacid-fast organisms will remain gram-positive in the face of acid alcohol as was seen in Experiment 6.

Why, when stained with Much's carbolfuchsin carbol gentian-violet stain, do the Much bodies appear deep purplish blue and not reddish? This occurs, as seen in this work, for the following reasons:

1. The gentian-violet obscures the fuchsin due to its darker coloration.

2. The alkali of the method of concentrating tubercle bacilli in sputum and pus interferes with the bright red of the fuchsin, not only of the Much method, but also of the Ziehl-Neelsen method. This probably accounts for the fact that Much "granules" have more frequently been demonstrated than tubercle bacilli with the Ziehl-Neelsen stain in pus from cold abscesses.

3. The nitric acid changes the color quality of the fuchsin to a lavender shade but has no apparent effect on the methyl violet.

In order to demonstrate that the so-called Much "granules" are actually a tuberculosis virus apart from the standard tubercle bacillus, it will be necessary for Much or the other advocates of this staining method to employ other than an acid-fast stain to demonstrate nonacid-fast bodies. As to what the real nature of the Much "granules" may he was not a part of the above work to ascertain, but these bodies greatly resemble granular tubercle bacilli or fragments thereof (see Fig. 1).

CONCLUSIONS

1. The Much staining technic is not a true or a modified Gram stain because it employs acid decolorizers instead of only alcohol as for the classical Gram stain.

2. It is an acid-fast stain because in it carbol dyes and acid alcohol decolorizers are employed. When the most important pathogenic acid-fast microorganism, the tubercle bacillus, is stained according to Much's technic, the latter is shown to be a strong acid-fast stain for this organism whose visibility is superior to that of tubercle bacilli stained with the Ziehl-Neelsen.

3. When the Much stain is applied to the granular form of the tubercle bacillus, the latter resembles, in fact is, indistinguishable morphologically from Much's granules. This work has not, however, endeavored to determine whether the Much "granules" are really tubercle bacilli or not.

4. If the "granular" bodies that are found in certain sputa and in pus from cold abscesses by the Much staining method are actually a different form of tuberculosis virus, one that is nonacid-fast and gram-positive, then this technic cannot be employed to demonstrate such a virus since it is an acid-fast staining procedure.

REFERENCES

1. Kretschmer, O. S.: The Gram Property of the Acid-fast Form of the Tubercle Bacillus, J. LAB. & CLIN. MED. **19**: 350, 1934.
2. Much, Hans: Berl. klin. Wehnschr. **45**: 700, 1908.
3. Todd and Sanford: Clinical Diagnosis by Laboratory Methods, ed. 6, 1929, p. 72. The seventh edition has deleted this description.
4. B. Möllers in Handbuch der pathogenen Mikroorganismen, by Kolle, Kraus, Uhlenhuth, 1930.

AN EVALUATION OF THREE METHODS FOR THE DEMONSTRATION OF TUBERCLE BACILLI FOR USE IN HOSPITAL ROUTINE*

A Special Study of Several Simple Culture Mediums

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IN RECENT years the efficacy of the culture method for the isolation and identification of the tubercle bacillus from various types of tuberculous material has received considerable notice in the literature. Corper and Uyei[1, 2] have reviewed the work of previous authors and published reports comparing the guinea pig inoculation method with the culture method. The question of the superiority of either method is still under discussion. Recent authors, Herrmann, Hansmann, and DeCapito,[3, 4] report superior results with guinea pig injections. At the same time, Norton, Thomas, and Broom[5] advocate the use of the culture method on the grounds of economy as well as efficiency.

The guinea pig inoculation method has been criticized because many guinea pigs contract tuberculosis spontaneously or have internal lesions which resemble those caused by the tubercle bacillus. Howell and Schultz[6] report an epizoötic among guinea pigs due to a paratyphoid B bacillus which caused lesions closely resembling tubercles in the spleen and liver. Magath and Feldman[7] in a recent complete review of the subject conclude that with reasonable care spontaneous tuberculous infection of guinea pigs can be avoided. Cummings[8] has reported the incidence of spontaneous tuberculous infection in laboratory animals in Saranac, a tuberculosis center.

In the present study an attempt has been made to compare direct smear, guinea pig inoculation and culture methods for the demonstration of the tubercle bacillus in the routine specimens received in the laboratory of a large hospital. This study differs from many previous ones in that no attempt was made to obtain material from clinically tuberculous patients. In hospital laboratories, the bacteriologist is more often concerned with ruling out a possible tuberculous infection rather than confirming a clinical diagnosis of tuberculosis. In many instances the diagnosis may be obscure or deferred until laboratory reports are complete. Therefore, the time element is important and must enter into a study of methods for the demonstration of the tubercle bacillus. As Feldman and Magath report,[9] animal inoculation remains the most satisfactory method for determining the virulence of an isolated acid-fast organism. However, if the condition of the patient indicates to the clinician the pathogenicity of the "unknown" causative organism, a quick laboratory report of an acid-fast bacillus of typical tubercle bacillus morphology and growth is of more value than a belated

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report of a virulence test. In this respect the culture method supplements identification of the tubercle bacillus by direct smear.

In some instances even a prolonged search fails to demonstrate tubercle bacilli in certain specimens. In that case, other methods of demonstration must supplement direct smears. Pottenger[10, 11] states that a search for five minutes yields 80 per cent of positive diagnoses obtainable in a fifteen-minute search; and that with careful work a longer search than fifteen minutes is not justified by the additional results. Von Huth and Lieberthal[12] state that smears were negative and cultures positive for the tubercle bacillus on 50 of 200 urines from tuberculous kidneys. They claim absolute reliability for the culture method. Corper,[13] realizing the difficulty of the quick demonstration of tubercle bacilli by direct smear when only small numbers of the bacilli are present in a specimen, has recently devised a tissue substrate culture method to supplement identification of the organism by direct smear.

TABLE I

THE DEMONSTRATION OF TUBERCLE BACILLI BY DIRECT SMEAR, GUINEA PIG INOCULATION AND CULTURE METHODS

CLINICAL DIAGNOSIS	MATERIAL	NUMBER OF SPECIMENS	DIRECT SMEAR	GUINEA PIG INOC.	CULTURE
Positive	Urine	1	+	+	+
	Tissue	3	+	+	+
	Pus	1	+	+	+
	Urine	3	−	−	+
	Pus	2	−	−	+
	Urine	2	+	−	−
	Spinal fluid	1	−	−	−
	Pus	1	−	+	+
	No. Positive		7	6	11
Negative	Urine	8	−	−	−
	Pus	3	−	−	−
	Fluid exudate	1	−	−	−
	Stools	2	−	−	−
	No. Positive		0	0	0
Unknown	Stools	3	−	−	−
	Fluid exudate	16	−	−	−
	Tissue	1	−	−	−
	Spinal fluid	3	−	−	−
	Sputum	3	−	−	−
	Pus	8	−	−	−
	Fluid exudate	1	−	+	−
	Urine	1	−	−	+
	Fluid exudate	1	−	−	+
	Tissue	1	−	+	+
	Spinal fluid	2	−	+	+
	Urine	1	−	+	+
	No. Positive		0	5	6

In the present study 69 specimens (Table I) were examined by direct smear, guinea pig inoculation and culture methods. Table I outlines these cases according to the clinical diagnoses. Direct smears were examined for thirty minutes. Fourteen specimens were obtained from clinically tuberculous patients. Of these, 5 specimens were positive by all three methods. In 2 cases, smears were positive and the results were not confirmed by either cultures or guinea pig injections; when examined after two months both cultures and

guinea pigs showed no evidence of tubercle bacilli. In 5 cases, demonstration of the tubercle bacillus was accomplished when both smears and guinea pig inoculations gave negative results. In one case, all three methods gave negative results, and in another case both guinea pig inoculations and culture methods yielded a positive result when direct smears had been negative. Of these specimens from clinically known tuberculosis cases, 7 were proved positive by direct smear, 6 by guinea pig inoculation and 11 by culture methods.

Fourteen specimens were obtained from clinical cases not diagnosed as tuberculosis. In addition to the negative clinical diagnosis, routine cultures showed the presence of other organisms. The 14 specimens were negative for tubercle bacilli when examined by all three methods.

Forty-one specimens were sent to the laboratory with no clinical diagnoses on the records. In four instances guinea pig inoculations and cultures gave a positive result although the direct smears were negative. In two other cases, the guinea pig injections and direct smears were negative, but tubercle bacilli were isolated by cultures. In one instance the guinea pig inoculation alone showed a positive result. In this series a total of 5 specimens gave positive results by guinea pig inoculation, 6 by culture and none by direct smear. Thirty-four specimens were negative for tubercle bacilli when tested by all three methods.

Table II summarizes these results. From these figures the inadequacy of direct smears for the demonstration of tubercle bacilli and the superiority of culture methods to the guinea pig inoculation method is obvious.

Many types of culture mediums for the cultivation of the tubercle bacillus have been devised. Most of them have a base of eggs or potatoes and are reinforced with glycerol. Corper and Uyei[14, 15] have reported excellent results with crystal violet potato cylinder medium. The efficiency of the egg mediums of Miraglia,[16] Hohn,[17] Lubenau,[18, 19] Dorset,[20] Sweany and Evanoff,[21] and more recently Herrold,[22] Feldman,[23] Woolley and Petrik,[24] has also been favorably

TABLE II

A COMPARISON OF DIRECT SMEAR, GUINEA PIG INOCULATION AND CULTURE METHODS FOR THE POSITIVE DEMONSTRATION OF THE TUBERCLE BACILLUS IN CLINICALLY POSITIVE, NEGATIVE AND QUESTIONABLE MATERIAL

METHOD OF DEMONSTRATION	NUMBER OF SPECIMENS	NUMBER OF POSITIVE SPECIMENS	PERCENTAGE OF POSITIVE RESULTS
Culture	69	17	24.6
Direct smear	69	7	10.1
Guinea pig inoculation	69	11	15.9

reported in the literature. Koch's[25] original serum medium has given way to synthetic mediums such as Long's.[26, 27] A simple medium with asparagin as a source of nitrogen has been devised by Lowenstein.[28, 29, 30] The asparagin is substituted for meat extracts which, he says, will not inhibit the growth of the tubercle bacillus. It is doubtful whether the ideal medium for the cultivation of the tubercle bacillus has yet been devised.

In routine laboratory procedure it is obviously impossible to employ a large number of mediums. In order to determine the most satisfactory ones, cultures were made on several well-known mediums using Corper's, Hohn's and Miraglia's

as controls. One specimen with a positive direct smear showed growth of tubercle bacilli in seven days on both Hohn's and Miraglia's mediums, but seventy-five days were required for growth to appear on Lubenau's egg medium. Four specimens were cultivated on Herrold's egg yolk agar. No growth was obtained on this medium or on the controls. Known cultures of tubercle bacilli grew very poorly when transferred onto Herrold's medium. In spite of careful precautions, tubes of Herrold's medium dried out early in the long period of incubation necessary for the growth of tubercle bacilli. This medium also showed a high percentage of contaminated cultures. These experiences substantiate the statement of Magath and Feldman[5] that Herrold's reports are not complete. Woolsey,[31] however, has found Herrold's medium an excellent means of demonstrating tubercle bacilli.

Woolley and Petrik's egg medium is rather complicated to prepare, but supports excellent growth and, therefore, may be used for stock cultures of tubercle bacilli. Of 36 specimens cultured on this medium and on the controls, two were positive on the controls and one on Woolley and Petrik's medium after twenty-five days. Nineteen of these same specimens were cultivated on 5 per cent glycerol agar, long a standard medium. The same specimens which showed growth of tubercle bacilli in twenty-five days on Woolley and Petrik's medium became positive in fifty days on the glycerol agar. Glycerol agar may be used for stock cultures of tubercle bacilli, but it dries out rapidly and often becomes contaminated.

Thirty-one specimens were cultured on Lowenstein's medium. Three of these specimens were positive on the controls, none on Lowenstein's medium. Eight blood samples were included on this series. Five of them were from patients with acute arthritis or rheumatic fever, one of them from a case of typhoid, and two from tuberculous patients. All eight specimens gave negative results on the controls as well as on Lowenstein's medium. This is contrary to Reitter and Lowenstein's[32] report that a high percentage of positive growth of tubercle bacilli is obtained when blood specimens from arthritic, rheumatic and tuberculous patients are cultured by Lowenstein's method on his medium. Although Abt[33] claims reliability for Lowenstein's method, Cummings[34] reports negative blood cultures on both Hohn's and Lowenstein's mediums. Weatherall[35] was unable to substantiate Lowenstein's work. Wallgren[36] states the necessity for specialized technic if positive blood cultures are to be obtained. Additional study of Lowenstein's medium in the present investigation proved that it supported only scanty growth of tubercle bacilli even when inoculated with large amounts of stock cultures.

For standard work, therefore, these various mediums were rejected in favor of Corper's, Hohn's and Miraglia's mediums. These do not require elaborate preparation and prove to be efficient.

MEDIUMS

Hohn's medium was made according to standard methods.[17]

Corper's medium was made according to his standard method.[14]

The preparation of Miraglia's medium is described here because this method differs in its extreme simplicity from standard methods described elsewhere.

Miraglia's medium originally consisted of seven egg yolks in 100 c.c. of a 5 per cent glycerol beef broth. More recently, in accordance with the work of Feldman,[23] a 6 per cent glycerol water solution has been substituted for the broth. However, no change in results has been noticed.

The eggs are sterilized by immersing in 60 per cent alcohol for ten minutes. Sterilize the hands, break the eggs quickly, separate the whites from the yolks, and drop the yolks into a large sterile flask with a wide mouth. If the flask is sufficiently large, the egg yolks will break as they hit the bottom and thus eliminate stirring, an obvious element in contamination. Previously sterilized and measured broth is then added, the mixture well shaken, tubed under sterile conditions and coagulated in an Arnold or oven at 90° C. Medium made by this simple method has always been found to be sterile.

PROCEDURE

All specimens except stools, sputums, and tissues were cultured and if sterile were inoculated without further treatment onto tubercle bacillus mediums. Contaminated specimens must be treated with some reagent. The more common ones in use are 5 per cent oxalic acid, 6 per cent sulphuric acid, 15 per cent sulphuric acid, 3 per cent sodium hydroxide, 3 per cent hydrochloric acid, 3 per cent acetic acid, or antiformin. In the present study one-half of the specimens were treated with 6 per cent sulphuric acid and the other half with 5 per cent

TABLE III

A COMPARISON OF CORPER'S, HOHN'S AND MIRAGLIA'S MEDIUMS FOR THE ISOLATION OF THE TUBERCLE BACILLUS

DIAGNOSIS	MATERIAL	NUMBER OF SPECIMENS	CORPER'S MEDIUM	HOHN'S MEDIUM	MIRAGLIA'S MEDIUM
Positive	Tissue	1	+	+	+
	Urine	4	+	+	+
	Urine	1	+	−	−
	Pus	1	+	−	−
	Pus	1	+	+	−
	Urine	1	−	+	+
	Pus	1	−	+	+
	Tissue	1	−	+	+
	Urine	1	−	−	−
	Sputum	1	−	−	−
	Blood	2	−	−	−
	Spinal fluid	1	−	−	+
	Tissue	1	−	−	+
	Pus	1	−	−	+
	Tissue	1	−	+	−
	No. Positive		8	10	11
Negative	Urine	7	−	−	−
	Sputum	1	−	−	−
	Blood	2	−	−	−
	Spinal fluid	1	−	−	−
	Pus	1	−	−	−
	Stools	3	−	−	−
	No. Positive		0	0	0
Unknown	Stools	2	−	−	−
	Pus	9	−	−	−
	Spinal fluid	2	−	−	−
	Fluid exudate	15	−	−	−
	Sputum	2	−	−	−
	Urine	32	−	−	−
	Pus	1	−	+	+
	Fluid exudate	1	+	−	−
	Urine	1	+	+	+
	Urine	1	−	+	−
	Urine	1	−	−	+
	No. Positive		2	3	3

oxalic acid. Both reagents appeared to give equally efficient results. Fifteen per cent sulphuric acid was used to kill the contaminating organisms in stools.

Stools, sputums, tissues, and contaminated materials were treated with equal parts of the reagent for one-half hour at 37° C. Solid specimens were ground in a mortar with the reagent. The treated material was then centrifugalized for one-half hour, washed with sterile physiologic saline solution, recentrifugalized and the sediment inoculated onto the mediums with a pipette. Blood specimens were treated according to Lowenstein's method before inoculation. Four or five drops of sediment were smeared over the surface of each tube of medium and at least four tubes of each medium were inoculated for each specimen. The tubes were sealed by pipetting approximately 2 c.c. of melted paraffin on each plug; the tubes were capped with tinfoil. All cultures were incubated in covered tin cans for four months at 37° C. in an incubator before being discarded. Smears were made periodically and again just before the cultures were discarded.

Table III summarizes the results obtained using Corper's, Hohn's, and Miraglia's mediums. Nineteen specimens were received from patients with clinical diagnosis of tuberculosis. Fifteen specimens yielded positive growth on at least one medium. Five were positive on all three mediums; four were negative on all three. Two of these four negative specimens did not produce tuberculosis when injected into the guinea pigs. Two were blood specimens which were not inoculated into guinea pigs. Three specimens were positive on Miraglia's alone, two on Corper's alone, and one on Hohn's alone. One specimen gave a positive growth on both Hohn's and Corper's mediums and three were positive on both Hohn's and Miraglia's. In this series a total of eight specimens gave positive growth on Corper's medium, ten on Hohn's medium, and eleven on Miraglia's medium.

Fifteen specimens from patients with a clinical diagnosis other than tuberculosis gave no growth of tubercle bacilli on the three mediums.

Sixty-seven specimens were received with clinical diagnoses unknown. Of these, 62 were negative. One specimen gave positive results on all three mediums, one each on each of the three mediums, one each on each of the three mediums alone, and one, on Hohn's and Miraglia's only.

These results would lead to the conclusion that Corper's medium is less favorable to the growth of the tubercle bacillus than are the other two mediums. Corper's medium, however, showed contaminations in only 5.2 per cent of tubes inoculated, Hohn's in 13.6 per cent, and Miraglia's in 19.4 per cent.

For further comparison of mediums a second series of 68 specimens were cultivated on Corper's medium and on Miraglia's medium. Table IV summarizes the results obtained in this study.

Five specimens were from tuberculous patients. Tubercle bacilli from one of these grew on both Corper's and Miraglia's mediums. One was positive on Miraglia's alone; 3 were negative on both mediums. Of the total 5, one was positive on Corper's and 2 on Miraglia's medium.

Six specimens tested were obtained from patients with a clinical diagnosis other than tuberculosis. All 6 specimens were negative for tubercle bacilli when cultured on both mediums.

Fifty-seven specimens were received with diagnoses deferred or unknown. Fifty-four were negative when cultivated on both mediums. One was positive on Miraglia's alone, and 2 were positive on both Miraglia's and Corper's

TABLE IV

A COMPARISON OF CORPER'S AND MIRAGLIA'S MEDIUMS FOR THE ISOLATION OF THE TUBERCLE BACILLUS

DIAGNOSIS	MATERIAL	NUMBER OF SPECIMENS	CORPER'S MEDIUM	MIRAGLIA'S MEDIUM
Positive	Tissue	1	+	+
	Tissue	1	−	+
	Tissue	1	−	−
	Spinal fluid	2	−	−
	No. Positive		1	2
Negative	Tissue	1	−	−
	Blood	3	−	−
	Pus	1	−	−
	Fluid exudate	1	−	−
	No. Positive		0	0
Unknown	Fluid exudate	8	−	−
	Blood	2	−	−
	Tissue	1	−	−
	Stools	6	−	−
	Spinal fluid	1	−	−
	Sputum	4	−	−
	Urine	30	−	−
	Pus	2	−	−
	Urine	1	+	+
	Spinal fluid	1	+	+
	Spinal fluid	1	−	+
	No. Positive		2	3

mediums. A total of 2 were positive on Corper's and 3 on Miraglia's medium. In this series the percentage of contaminated cultures was approximately 5 per cent for Corper's and 10 per cent for Miraglia's medium.

From these results it is apparent that the egg mediums are more sensitive than Corper's medium for the isolation of tubercle bacilli. However, since Corper's medium becomes contaminated much less often than Hohn's or Miraglia's, it should be included as a control medium in routine cultures.

As has been emphasized, the time element is important in the laboratory demonstration of tubercle bacilli. Positive results were obtained by guinea pig injection occasionally in twenty-one days, but the average period was sixty days. Cultures on egg mediums were quite often macroscopically positive in seven days; the average number of days in which positive cultures were ob-

TABLE V

METHOD	MINIMUM NUMBER OF DAYS FOR GROWTH TO APPEAR	AVERAGE NUMBER OF DAYS IN WHICH GROWTH APPEARED
Positive guinea pig inoculations	21	60
Positive cultures on Corper's medium	25	38.5
Positive cultures on Hohn's medium	7	27.7
Positive cultures on Miraglia's medium	7	30.7

tained on Hohn's medium was 27.7 days and on Miraglia's 30.7 days. This substantiates the previous statement that the low percentage of contaminated cultures obtained with Corper's medium is the most important reason for its use in routine work. These statements are summarized in Table V.

SUMMARY AND CONCLUSIONS

A comparison of direct smear, guinea pig inoculation and culture methods for the demonstration of tubercle bacilli in various types of specimens indicated culture methods to give a greater percentage of positive results more quickly than either of the other two methods.

A study of various mediums for the isolation of the tubercle bacillus proved the three simple mediums, Corper's, Hohn's and Miraglia's, to be suitable for routine diagnostic cultures. Hohn's and Miraglia's mediums gave larger numbers of positive results in less time than did Corper's medium. Either of these two mediums may be used with equal efficiency. Corper's medium, however, is sufficiently sensitive and becomes contaminated less often than the egg mediums and, therefore, should be included in routine culture methods.

REFERENCES

1. Corper, H. J., and Uyei, Nao: The Cultivation of Tubercle Bacilli. An Improved Method for Isolation from Tuberculous Materials, J. LAB. & CLIN. MED. **13**: 469, 1928.
2. Corper, H. J., and Uyei, Nao: Further Observations with a New Method for Cultivating Tubercle Bacilli: A Comparison with Guinea-Pig Inoculation and Petroff's Method, J. LAB. & CLIN. MED. **14**: 393, 1929.
3. Herrmann, W. W., Hansmann, G. H., and DeCapito, Thelma: Demonstration of Mycobacterium Tuberculosis in Exudates, Tissues and Body Fluids, Proc. Soc. Exper. Biol. & Med. **29**: 668, 1932.
4. Herrmann, W. W., Hansmann, G. H., and DeCapito, Thelma: The Demonstration of Mycobacterium Tuberculosis in Exudates, Tissues and Body Fluids. Concerning Guinea-Pig Inoculation and Cultural Method for the Demonstration of Mycobacterium Tuberculosis, Am. J. Clin. Path. **2**: 371, 1932.
5. Norton, John F., Thomas, James G., and Broom, Norma H.: Laboratory Tests for Tubercle Bacilli by Culture Methods, Am. Rev. Tubere. **25**: 378, 1932.
6. Howell, Katharine M., and Schultz, Oscar T.: An Epizootic Among Guinea-Pigs Due to a Paratyphoid B. Bacillus, J. Infect. Dis. **30**: 516, 1922.
7. Magath, Thomas B., and Feldman, W. H.: The Relative Value of Cultural Methods and Guinea-Pig Inoculation in the Diagnosis of Tuberculosis, Am. J. Clin. Path. **2**: 199, 1932.
8. Cummings, Donald E.: Naturally Acquired Tuberculous Infection Among Saranac Laboratory Guinea-Pigs, Am. Rev. Tubere. **26**: 388, 1932.
9. Feldman, W. H., and Magath, T. B.: The Reliability of Guinea-Pig Inoculation in the Diagnosis of Tuberculosis, Am. Rev. Tubere. **24**: 312, 1931.
10. Pottenger, Joseph E.: The Importance of the Time of Search in Examining Stained Preparations for Rare Tubercle Bacilli, J. LAB. & CLIN. MED. **16**: 985, 1931.
11. Pottenger, Joseph E.: The Demonstration of Rare Tubercle Bacilli in Sputum, Am. Rev. Tubere. **24**: 583, 1931.
12. von Huth, Theodore, and Lieberthal, Frederick: The Culture of Tubercle Bacilli from the Urine, Surg. Gynec. Obst. **50**: 985, 1930.
13. Corper, H. J.: A Tissue Substrate Microculture for Tubercle Bacilli, J. A. M. A. **99**: 1913, 1932.
14. Corper, H. J., and Uyei, Nao: A Simple Glycerol Water Crystal Violet Potato Cylinder Medium for Diagnostic Cultures of Tubercle Bacilli, Arch. Path. **7**: 835, 1929.
15. Corper, H. J., and Uyei, Nao: Oxalic Acid as a Reagent for Isolating Tubercle Bacilli and a Study of the Growth of Acid-fast Nonpathogens on Different Mediums With Their Reaction to Chemical Reagents, J. LAB. & CLIN. MED. **15**: 348, 1930.
16. Miraglia, M.: Sull 'Importanza della Coltura del Bacillo di Koch nella Diagnosi della Tubercolosi, Pediatria **37**: 1167, 1929.
17. Hohn, Joseph: Die Kulture des Tuberkelbazillus sur Diagnose der Tuberkulose, München. Med. Wehnschr. **73**: 2162, 1926.
18. Lubenau, C.: Der Eigelnahrboden als Ersatz des Serums zur Kultur von Diphtherie und Tuberkelbazillen Hyg. Rundschau, Berlin **17**: 1456, 1907.
19. Lubenau, C.: Lubenau's Egg Medium cited in Stitt, E. R.: Practical Bacteriology, Blood Work and Animal Parasitology, ed. 8, Philadelphia, 1927, P. Blakiston's Son & Co.
20. Dorset, M.: The Use of Eggs as a Medium for the Isolation and Cultivation of the Bacillus Tuberculosis, Am. Med. **3**: 555, 1902.

21. Sweany, H. C., and Evanoff, M.: Further Studies on the Cultivation of the Tubercle Bacillus, Am. Rev. Tuberc. **18**: 661, 1928.
22. Herrold, R. D.: Egg Yolk Agar Medium for the Growth of Tubercle Bacilli, J. Infect. Dis. **48**: 236, 1931.
23. Feldman, W. H.: Simplified Egg Medium for Cultivation of Mycobacterium Tuberculosis, Am. Rev. Tuberc. **26**: 317, 1932.
24. Woolley, J. Stanley, and Petrik, Frank G.: A Potato-egg Medium for the Isolation of Tubercle Bacilli, Am. Rev. Tuberc. **24**: 596, 1931.
25. Koch, Robert: Die Aetiologie der Tuberkulose. In: Mitth. a.d.k. Gandhtsamte. Berlin, August Hirschwald **2**: 88, 1884.
26. Long, E. R.: A Study in Fundamentals of Nutrition of the Tubercle Bacillus: The Utilization of Some Amino Acids and Ammonium Salts, Am. Rev. Tuberc. **3**: 86, 1919.
27. Long, E. R., and Seibert, F. B.: The Chemical Nature of the Active Principle of Tuberculin, Tubercle **8**: 111, 1926.
28. Lowenstein, E.: Ueber die Reinkulturmethode des Tuberkelbazillus, Wien. med. Wchnschr. **77**: 767, 1927.
29. Lowenstein, E.: Ueber Reinzuchtung von Tuberkelbazillen aus dem Blute, Wien. klin. Wchnschr. **43**: 294, 1930.
30. Lowenstein, E.: Die Methodik der Reinkulture von Tuberkelbazillen aus dem Blute, Deutsche med. Wchnschr. **56**: 1010, 1930.
31. Woolsey, C. I.: Diagnosis of Tuberculosis; Comparison of Results Obtained with Herrold Egg Yolk Agar Medium and with Inoculation of Guinea-Pigs, J. Infect. Dis. **49**: 177, 1931.
32. Reitter, C., and Lowenstein, E.: Akuter Gelenkrheumatismus und Tuberkelbazillamie, München. med. Wchnschr. **78**: 2133, 1931.
33. Abt, Frieda: Ueber den Kulturellen Nachweis der Tuberkelbazillen in Blute. Untersuchungen an Meerschweinchen und an Menschen, Schweiz. med. Wchnschr. **45**: 1083, 1931.
34. Cummings, John N.: The Cultivation of Tubercle Bacillus and Occurrence of Tuberculous Bacillaemia, Lancet **1**: 983, 1932.
35. Weatherall, Cicely: Tubercle Bacilli in the Blood Stream in Human and Animal Tuberculosis, Lancet **1**: 980, 1932.
36. Wallgren, Arvid: Einige Untersuchungen über Tuberkelbazillamie ad Modum Lowenstein, Arch. f. Kinderh. **95**: 297, 1932.

MODIFIED TECHNIC FOR MAKING WRIGHT'S BLOOD STAIN*

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THE object of this note is to suggest a slight modification in the technic for making the usual Wright's stain of a blood film. The apparatus requires two staining jars with covers. The reagents are pure methyl alcohol and Wright's stain† diluted up to 30 per cent with distilled water.

One staining jar is filled with pure methyl alcohol. The second contains the 30 per cent solution of Wright's stain. The blood film is made and dried in the usual manner. It is then immersed in methyl alcohol for at least five minutes. The slide is then placed in the stain for from two or three minutes up, depending upon the intensity of the stain desired. The slide is then washed as usual.

Using this technic there is no precipitation on the slide. It has been found that this method is economical. The stain can be used repeatedly instead of only once as in the usual technic. When the fluid level of the reagents becomes low more is added until there is enough to cover the slide. It is not necessary to discard any of the stain. Even though the solutions are used for several months staining takes place with good results. Even though the slide is in the methyl alcohol for an hour or more, there is no change in the staining properties of the cells.

*From the Pyengyang Union Christian Hospital.
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†The Wright's stain used in this study was prepared by dissolving one tabloid (Burroughs Welcome Co.) in 10 c.c. pure methyl alcohol.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

DIPHTHERIA: A Differential Culture Medium for B. diphtheriae and B. pseudodyphtheriae (Un Nuovo Metodo Culturale per la Differenzazione Del Bacillo Difterico Dai Bacilli Pseudodifterico), Bruschettini, G. Diagn. E. Techn. di Lab. 4: 212, 1933.

On this medium the diphtheria bacillus, fermenting glucose, produces a greenish color; in the absence of glucose-fermenters the medium shows a yellowish tinge. Because of its lack of specificity, the method is not reliable for direct isolation.

Preparation of Medium.—

1. Immerse a fresh egg in 1:1000 bichloride of mercury solution for 1 hour.
2. Place the egg "in alcohol" (per cent not stated) for 2 hours.
3. Dip one end of the egg in ether which is then burned off in the flame.
4. With a sterile pipette puncture the shell and transfer the contents to a sterile, glass-stoppered bottle (150 c.c.).
5. Add an equal volume of sterile "creamless" (skimmed?) milk and shake vigorously for ten minutes.
6. To 60 c.c. of this mixture add the following with sterile precautions.

Beef serum, sterile	60 c.c.
Glycerin	5 c.c.
2% sterile solution of glucose	5 c.c.
2% sterile solution malachite green	5 c.c.

7. Mix by shaking five minutes, place in sterile tubes, slant, and coagulate in an inspissator.

VACCINES: Comparison of Relative Values of the Intracutaneous Skin Test and of the Pathogen-Selective Culture in Selecting Bacteria for Vaccines From Mixed Cultures, Solis-Cohen, M. Am. J. Clin. Path. 3: 305, 1933.

There is practically no difference between the reactions produced by intracutaneous injections of organisms that are able to grow in the patient's fresh, whole, coagulable blood and the reactions produced by organisms that are killed by his blood.

There seems to be no correspondence between absence of bactericidal power in the blood of the host against a given organism and production in that host of a positive reaction by the intracutaneous injection of such organism.

There probably is no relationship between hypersensitiveness in the host to the exogenous and endogenous toxins of a given organism and the pathogenicity of such organism for that host.

It is questionable whether intracutaneous tests can identify, in a mixed culture, the bacteria that are infecting the patient.

Therefore, intracutaneous skin tests are probably unreliable for selecting bacteria in the preparation of vaccines.

MILK, A Study of the Brom-Thymol-Blue Reaction in Freshly Drawn, Bryan, C. S. Am. J. Pub. Health 23: 721, 1933.

Apparently there are normal physiologic factors that are responsible for a variation in the PH of freshly drawn milk.

The test cannot be used alone to detect cases of mastitis, since positive reactions were obtained in animals that were always free of mastitis. No definite cause is given for these

positive reactions. The work of Hucker, et al., was confirmed, indicating that a negative brom-thymol-blue reaction does not assure freedom from mastitis.

The brom-thymol-blue reaction is of value as an aid in determining the relative disease or freedom from mastitis of a herd. If a large number of positive reactions are obtained it is a good indication that something is wrong in the herd but the individual cases cannot be detected.

PHOTOGRAPHY: The Invisible Photographic Screen, Harding, F. R. J. Biol. Photo. Assn. **1**: 203, 1933.

At the Children's Hospital has been developed what might be termed the "invisible screen," because the patient never sees it. It consists of a piece of thin celluloid the same size as the negative that is to be used. This sheet of celluloid is scored with cross-hatched lines one-quarter inch apart. The scoring is done with a scribing tool or the point of a knife. Care must be taken not to cut too deeply or the celluloid will split if it is bent. After the scoring is completed, red or black wax crayon is rubbed into the lines, and the sheet polished with a soft cloth.

To use the screen, lay it on the glass of the printing machine, place the negative on *top* of it and make the print in the usual manner. The result will be a positive covered with fine white, crossed lines.

As a guide in registering the screen with the negative, a thin, true vertical line is made on the background. This line should show somewhere on the negative. When ready to print, simply register the background line with one of the screen lines.

TUBERCLE BACILLI, Nutrient Quality of Eggs for Growing, Corper, H. J., and Cohn, M. L. Am. J. Hyg. **18**: 1, 1933.

Quantitative evaluation of inspissated egg yolk as contrasted with the inspissated white of egg shows the latter to be a poor nutrient for small numbers of tubercle bacilli as compared to the former. The inspissated yolk has also proved slightly superior to the inspissated whole egg.

The addition of broth is not essential to the nutrient qualities of whole egg or egg yolk media. The inspissation of egg or egg yolk media is beneficial to the nutrient qualities and insures sterility as compared to the use of raw unsterilized whole egg or egg yolk.

The addition of a non-nutrient substance such as agar does not introduce any beneficial action upon the nutrient qualities of whole egg or egg yolk media, and if added in too great amount diminishes their nutrient value for small numbers of bacilli.

The addition of water up to 25 per cent to egg yolk before inspissation serves to fulfil the purpose of preventing drying of this medium during the long period of incubation required for the growth of tubercle bacilli.

Mixing of two good nutrients for tubercle bacilli such as egg yolk and potato does not serve to enhance the nutrient qualities of either of these substances but may change the type of colony.

Large plantings of tubercle bacilli grow on a wide range of acidity in good nutrient media such as egg yolk, but a few bacilli require an acidity of narrower range approximating the neutral point.

The value of dyes intimately mixed, with a nutrient such as egg yolk is mainly to assist in the ease of finding early colonies, but they must be used cautiously in a concentration which exerts no tuberculostatic action in graded planting tests. Several pigments and dyes are adapted to this purpose.

SERUM CALCIUM, In Normal Boys, Molitch, M., Weinstein, S., and Cousins, R. F. Am. J. M. Sc. **186**: 378, 1933.

The serum calcium findings on 511 normal boys, between the ages of eight and twenty years, range from 7.6 to 12.5 mg. per cent. Of the total number reported, 33 were found

below and 1 above the extreme normal limits of 8.6 and 12 mg. per cent. Disregarding the highest and lowest 5 per cent of distribution, the normal limits are from approximately 8.5 to 10.8 mg. per cent. No progressive decrease was found with age in this group. Nationality and race showed no influence on serum calcium metabolism.

BLOOD CHEMISTRY: Serial N P N Studies and Their Prognostic Significance in Acute Coronary Occlusion, Steinberg, C. L. Am. J. M. Sc. **186:** 372, 1933.

From a study of 16 cases it is concluded that (a) a rising nonprotein nitrogen or (b) a nonprotein nitrogen that remains elevated in the blood plasma is of ill omen.

More attention should be paid to blood chemistry studies during coronary occlusion as a prognostic aid.

A lowering of the blood pressure and diminution in urine output will not alone explain the rise in nonprotein nitrogen in acute coronary occlusion.

HYPERGLYCEMIA, Hypertension, Obesity and, Musser, J. H., and Wright, D. O. J. A. M. A. **101:** 420, 1933.

A group of thirty obese hypertensive women exhibited a marked lowering of sugar tolerance. A second group of fat individuals without hypertension are shown not to have a hyperglycemia. It seems reasonable to assume that there is no one factor definitely responsible for the combination of obesity, hypertension and hyperglycemia unless it is obesity. The reduction in weight is often associated with a lowering of the blood pressure and a return of a sugar tolerance curve to normal.

PNEUMONIA, Value of Skin Test With Type-Specific Capsular Polysaccharide in the Serum Treatment of Type I Pneumococcus Pneumonia, Francis, T., Jr. J. Exper. Med. **57:** 4, 617.

Skin tests were made with Type I S.S.S. in 53 cases of Type I pneumococcus lobar pneumonia, 48 of which were treated with antipneumococcus Type I serum. In all but 1 of the 46 recovered cases a positive, immediate skin reaction was obtained at about the time of recovery. In 7 fatal cases reactions were consistently negative, even in the presence of circulating type-specific antibodies.

The skin test has proved to be an extremely valuable guide to serum therapy, and a definite prognostic aid. The test has distinct advantages over the agglutination reaction in that it is not merely an index of circulating antibodies. When positive, it invariably denotes that recovery has begun; when negative, it indicates further serum therapy. The mechanism of the positive skin test is closely related to that operative in recovery from pneumonia, and is apparently the resultant of antibody and tissue activity.

TUBERCULOSIS, Gastric Examination in Pulmonary Tuberculosis With Negative Sputum Examination, Ulmar, D., and Ornstein, G. G. J. A. M. A. **101:** 835, 1933.

A search was made of the gastric contents for tubercle bacilli in pulmonary tuberculosis. The mechanism of bronchial peristalsis is demonstrated whereby bronchial contents are raised to the level of the larynx without the mechanism of cough. The importance of this mechanism in raising sputum from the lungs is amply shown by the fact that, in a series of 287 cases in which there were repeated negative sputum examinations, approximately 20 per cent yielded tubercle bacilli on examination of the gastric contents. In some of those positive cases there was no previous coughing and expectoration. The importance of this method of investigation becomes increasingly significant when the normal cough mechanism has been interfered with as, for example, in pneumothorax or thoracoplasty cases. The simplicity of the examination, which consists merely in the usual concentration technic on the aspirated gastric contents, makes the examination so easy as to warrant its routine use in all suspected sputum negative cases.

HEPATIC FUNCTION: Clinical Values of Levulose and Galactose Tolerance Tests for, Radhakrishna, M. V. Ind. J. M. Res. 21: 141, 1933.

From this study it is concluded that the levulose tolerance test gave positive results in a greater number of cases with liver disease than in those without evidence of hepatic disorder. In some cases with definite evidence of liver disease the test gave negative results, and hence it is concluded that the test cannot be considered to be of diagnostic importance in any individual case.

The variable results obtained by the levulose tolerance test in cases of cirrhosis of the liver are considered to be due to the regeneration of the hepatic tissue. It is pointed out that a negative test in these cases cannot exclude liver disease, as it only indicates that a sufficient amount of actively functioning liver tissue is still left to maintain the carbohydrate metabolism. The test becomes positive only when there is diffuse destruction of the hepatic parenchyma together with failure of regeneration.

Though the galactose tolerance test of Bauer has its own advantages, it is misleading to depend on the urinary findings alone and the total amount of galactose excreted in the urine is too variable to consider it as a guide to hepatic efficiency.

ENDO MEDIUM, Nature of Reaction of Colon Organism on, Margolena, L. A., and Hansen, P. A. Stain Techn. 8: 131, 1933.

Considerable confusion in the understanding of Endo's reaction is caused by the fact that a faint coloration may be produced by weak acids and by oxidations, both causing a restoration of the fuchsin color.

The viewpoint of Neuberg and Nord that the cultivation on the Endo medium is a trapping procedure (Abfangverfahren) for acetaldehyde is confirmed.

The typical reaction of *Bacterium coli* is caused not by lactic acid, but by the intermediate product acetaldehyde which is fixed by sodium sulphite.

The color produced in the reaction is not restored fuchsin, which is shown by the fact that it cannot be shaken out with ether when picric acid has been added beforehand.

TYPHOID CARRIERS, Serological Diagnosis of, Wyllie, J. Am. J. Hyg. 78: 393, 1933.

In any agglutination method using living bacterial suspensions the pitfalls are due to the S R variation and to the H O variation. The former variation is easily detected by the appearance of spontaneous agglutination (granular in type) in increasing strengths of saline controls. A slight granularity in 0.85 per cent saline is more marked in 1.7 per cent and more so in 3.4 per cent and 6.8 per cent saline. When this occurs the stock culture is replated on agar and a smooth colony picked off. In this way the colony form is taken as an indication of smoothness or roughness and this is afterward examined for physiologic smoothness or roughness.

The latter variation (i.e., H O) is guarded against by subculturing the "H" variants on agar slants containing some water of condensation. O variants may be subcultured on dry agar slopes. The agar medium must be carefully prepared as outlined on pages 395 and 396 and the use of motility tubes containing semisolid agar ensures maximum development of the H-receptors. Felix (1931) recommends however that agar stabs for maintaining stock cultures over long periods should not contain muscle sugar, hence this agar should be prepared from meat extracts instead of fresh meat.

In this study of the serologic reactions of typhoid carriers by Felix' method, the sera were examined qualitatively with living bacillary suspensions of "H" and "O" strains of *B. typhosus* and *B. para-typhosus* A and B. In the large majority of these sera "O" agglutinins were found exclusively. The typhoid H suspension of the Oxford Standards Laboratory, however, revealed the presence of "H" agglutinins in nearly all of these sera.

The use of living typhoid and paratyphoid strains necessitated particular care to maintain the cultures in the optimum phase. The results show that sufficient differences in the type and degree of agglutination in the three classes of sera studied—from typhoid

carriers, from typhoid fever cases and from individuals receiving prophylactic inoculation—justify the opinion that an additional serological procedure is available to assist in distinguishing carriers from normal and from inoculated individuals.

TUBERCULOSIS, Effect of Exercise on the Leukocyte Count in Pulmonary, Kaminsky, J. Am. J. M. Sc. **186**: 484, 1933.

In 50 tuberculous patients leucocyte counts were made after one hour's rest in bed and the results compared with those of blood counts taken after some exercise.

Thirty-eight cases showed an increase in their total counts following exercise (increases of more than 2,000 cells were obtained in 19 cases); 12 cases showed a decrease in their total counts taken after exercise.

Striking variations were noted in some of the differential counts taken after exercise; increases in the cells of some types exceeding 100 per cent were encountered.

The value of performing leucocyte counts under basal conditions in the study of cases with pulmonary tuberculosis is emphasized.

RETICULOCYTES: A New Method of Enumeration, Schleicher, E. M. Am. J. Clin. Path. 3: 395, 1933.

Solution A

Neutral potassium oxalate	1.0	gram
Sodium chloride c. p.	0.85	gram
Distilled water	100	c.c.

Solution B

Brilliant cresyl blue	1.0	gram
Sodium chloride c. p.	0.85	gram
Distilled water	100	c.c.
Chloretone (as a preservative)	1.0	gram

In a conical tipped centrifuge tube place 25 parts of Solution A and 5 parts of Solution B, mix (since a precipitate forms when the solutions are mixed, it is better to make up a larger volume, filter and place the proper amount of the mixed solution in the tube and proceed), and add several drops of blood. After thoroughly mixing, permit to stand ten to twenty minutes, then centrifugalize for twenty to thirty seconds at a moderate speed. The supernatant fluid is pipetted off until a layer of fluid approximately equal to the depth of the sediment remains. Mix the sediment well with the supernatant liquid, draw up in the pipette and discharge one drop near the end of a perfectly clean glass slide.

Spread the stained drop with the edge of a coverslip square 18 mm. and draw the film to about 6 cm. from the starting point.

The underlying principle of the method involves the Relative Reticulocyte Distribution (R. R. D.) throughout the smear. To know the relative distribution of reticulocytes is important, since it is technically not possible to make a blood film manually so that the distribution of erythrocytes and reticulocytes is equal throughout the preparation. It has been determined, however, that the distribution varies in a definite way when the above technic of preparing smears is followed each time.

The R. R. D. is found as follows: Divide a piece of paper into 10 columns and mark them 1 to 10. Adjust a 4× eyepiece to a tube-length of 190 mm., using an oil immersion objective 1.8 mm. × 95 N.A. 1.30 in order to obtain the specific microscopic working field. Any other combination giving the same field may be used.

The R. R. D. in the film will usually be found to show about four variations in concentrations as follows: (1) extremely high, (2) high, (3) low, (4) extremely low or absent.

Place the objective on the upper edge of the film near the starting point, that is, the extreme left border of the film. Moving the slide upward in a vertical line, count several

fields that show the highest as well as the moderately high, moderately low and low reticulocyte concentrations. Record the counts of each classification in column 1. When the lower edge of the film is reached, move the slide to the left horizontally 0.5 cm. (measured by the mechanical stage), from this point move the slide downward, count the reticulocyte concentrations as before and record them in column 2. Count ten vertical zones in this manner. If a vertical zone does not show any reticulocyte, mark in the corresponding column a zero.

In a vertical zone 18 mm. in length there will be found approximately 150 microscopic fields, the average field showing about forty erythrocytes. There should be counted and recorded in each column the number of reticulocyte concentrations found in at least eight to ten fields in each vertical 18 mm. zone. Select from the 80 to 100 individual enumerations recorded in the ten columns ten reticulocyte counts distributed as follows:

(a) Two fields showing extremely high concentrations.

(b) Three fields showing high concentrations.

(c) Two fields showing low concentrations.

(d) Three fields showing extremely low to absent concentrations (when selecting the individual reticulocyte counts use as many zeros as there have been zero columns).

Having added the ten reticulocyte counts the total number is referred to as the sum of reticulocytes (SR). In the same ten fields there are 400 erythrocytes. The ratio of reticulocytes to erythrocytes is therefore $\frac{SR}{400}$

The percentage of reticulocytes is $\frac{SR}{400} \times 100$

or per cent $= SR \times .25$

TUBERCULOSIS, Sanocrysin Treatment, Henrichsen, K. J., Sweany, H. C., and Hruby, A. J. Am. Rev. Tubere. (Suppl.) **28**: 1, 1933.

About 50 per cent of tuberculous patients having a stationary or downward progressive advanced tuberculosis of the "B" or "C" types, when given Sanocrysin in well-regulated doses, will show (1) a rather prompt cessation of symptoms (drop of temperature and pulse, increase of weight, and improvement of general condition), which changes tend to be permanent if the patient observes the usual measures of hygiene; (2) a clearing of tuberculous infiltration, with marked fibrosis and contraction of cavities, as shown by roentgenograms; and (3) changes from unfavorable to favorable laboratory findings.

The remainder of the treated patients may be divided about equally into three classes: those who show a temporary improvement for a few weeks to a few months; those who show no favorable change; and those who will not tolerate the drug at all.

By all the methods of control available and carefully analyzed figures, we can conclude that there is a shortening of the convalescent period or prolongation of life or both in the treated patients of Group 1.

There seems to be a real indication for the drug's use as a supportive treatment in collapse therapy when patients are not doing well, and when there is a beginning spread to the other side, or when there is need to clear up a slight involvement of one side in preparation for collapse therapy on the other.

Sanocrysin may be used to speed the convalescence of patients with favorable prognosis, but this is usually unnecessary.

The drug may be added to the armamentarium of the tuberculosis specialist for use when other measures are unavailable, as well as for other "reacting" types when the skill of the physician is able to direct a successful course.

More experiment is warranted, so that indications, or contraindications, and more favorable types may be better established, in all ages, stages, complications, types of the disease, and in both sexes.

AGGLUTININS: The Agglutinin Content of the Blood Following Typhoid and Paratyphoid Immunization, Foord, A. G., and Forsyth, A. Am. J. Clin. Path. 3: 333, 1933.

Agglutination tests conducted simultaneously with the same flask of antigen on serums of 120 nurses and internes immunized by three weekly subcutaneous injections of triple typhoid paratyphoid A and B vaccine furnished by the New York State Department of Health revealed the presence of typhoid agglutinins in a dilution averaging 1:320 to 1:640 at the end of twenty-three days following the last injection.

Tests at longer intervals of several months and to from five to nine years demonstrated persistence of agglutinins in all the serums studied in lower, but clinically significant amount; for example, 1:160 after six and nine years, respectively.

Agglutinins against paratyphoid bacilli were demonstrated at titers averaging somewhat lower than the corresponding titer against the typhoid bacillus.

In evaluating a positive agglutination test against typhoid or paratyphoid organisms, the history of prophylactic immunization must be considered, even though many years have elapsed since the inoculations.

PREGNANCY: The Rabbit Ovulation Test, Anklesaria, S. B. J. Univ. of Bombay **1:** 108, 1933.

All the biologic tests of pregnancy depend on the presence, in the body, of living chorionic tissue in biologic contact with the blood stream.

Pregnancy is a physiologic condition in which chorionic tissue is present and is in contact with the blood stream; but should a man or woman be the unfortunate possessor of a tumor containing chorionic tissue, i.e., a chorionepithelioma, he or she should give a positive biological test. Thus its use in the diagnosis of these tumors both in the female and in the male in the testis for example, is quite apparent.

In normal pregnancy the test is positive as early as three weeks after conception. It becomes negative in two to seven days after puerperium, the rabbit ovulation test becoming negative earlier (forty-eight hours).

In abnormal pregnancy the tests are useful in the diagnosis of ectopic gestation, abortions in various phases, and hydatidiform mole.

In ectopic gestation the test remains positive so long as there is a certain amount of living chorionic tissue in biologic contact with the maternal tissues. The author found it repeatedly useful and in only one case out of seven was it negative; this case being of long standing with no living chorionic tissue. In such cases, however, the danger of ectopic gestation is much less. The author shall, however, emphasize the fact that a full dose of 24 c.c. of urine and a forty-eight-hour interval between the first injection and the laparotomy will go a great way in diminishing the number of negative results.

In cases of death of the ovum in uterine pregnancy, the reaction takes some time to become negative, the time being greater in advanced gestation; it may take as long as a month or more. The significance of a positive reaction becoming negative is great in diagnosing death of the ovum.

Though the reaction disappears within forty-eight hours or a little more in the normal puerperium, it takes two months or a little more to disappear after the passage of a hydatid mole, though the mole may not be malignant.

In cases of hydatid mole and chorionepithelioma, the author has found the test positive with very small amounts. He is determining at present the smallest quantity of urine that would give a positive reaction in normal pregnancy, which work will be published in a future paper.

TUBERCLE BACILLI, Heavy Drop Method for Demonstration of, in Sputum, Kornetov, I. Klin. Med. Moscow **11:** 109, 1933.

A particle of the suspected sputum is placed in the center of the glass slide. The amount is determined by its consistency. If the sputum is fluid, a particle about 10 mm. in diameter may be used; if it is thick, a particle of a diameter not exceeding 5 mm. is

used. Five drops of a mixture of 20 c.c. of 10 per cent ammonium hydroxide solution and 30 c.c. of 96 per cent alcohol are added. By means of a thin glass rod the sputum and solutions are mixed. In a few minutes five more drops of the mixture are added as before. If a homogeneous mass has not been obtained, five more drops are added. After five minutes the mass is usually homogeneous. The mixture is then dried in an autoclave or an incubator. The preparation dries in the air in two to three hours, under proper temperature in about twenty minutes. Excessive heating should be avoided. The dried mass is stained without fixation. Sufficient carbolfuchsin to cover the mass is added. The slide is heated until it steams; this procedure is repeated twice and the carbolfuchsin is decanted. The preparation is treated repeatedly with 5 per cent nitric or sulphuric acid, the acid being washed off each time, so that finally the preparation assumes a slight rosy color. The preparation is then treated with a few drops of a 1 to 500 solution of methylene blue or a few drops of a 1 to 500 solution of chrysoidin. The slide is washed and dried.

By this method each field contains from 6 to 20 times more tubercle bacilli, if such are present, than the usual smear.

REVIEWS

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

The History and Epidemiology of Syphilis*

THIS book is a series of three lectures delivered under the Adolph Gehrmann endowment at the University of Illinois College of Medicine in March, 1933. The first lecture discusses the origin of the disease, presenting the evidence for and against its importation into Europe by the sailors of Columbus. The second lecture summarizes the historical development of our understanding of the nature of the disease and its treatment. The third discusses present epidemiologic problems and outlines preventive possibilities. For further discussion see editorial in this number.

The History of Dermatology†

IT IS only within the last hundred years that dermatology has become a specialty. As the author remarks, the history of dermatology, especially prior to 1800, is the history of medicine in general. He has, however, abstracted from the history of medicine the points that are of particular interest in connection with dermatology and the first sixty odd pages are devoted to the facts of dermatology as they have been developed in the history of medicine up to the beginning of the nineteenth century. The second two-thirds of the book deals with the historical development of dermatology as a specialty. This has been done in a very readable and informative way. Sometimes the reader wishes the author had more space to devote to the development of the personalities of the men who contributed so much to the specialty.

A distinct innovation, as an appendix, is the author's historical index on dermatology. In it he lists the various dermatologic diagnoses alphabetically, with brief summaries describing when such diseases were first mentioned in history, with other outstanding historical points about them.

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*The History and Epidemiology of Syphilis. The Gehrmann Lectures, University of Illinois. 1933. By Wm. Allen Pusey, A.M., M.D., LL.D., Professor of Dermatology Emeritis, University of Illinois, Sometime President of The American Dermatological Association and of The American Medical Association. Cloth, pages 113. Springfield, Ill. Charles C. Thomas, 1933.

†The History of Dermatology. By Wm. Allen Pusey, A.M., M.D., LL.D., Professor of Dermatology Emeritus, University of Illinois; Sometime President of The American Dermatological Association and of The American Medical Association. Illustrated, cloth, pages 223. Springfield, Ill. Charles S. Thomas, 1933.

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EDITORIAL

The Origin of Syphilis

TWENTY years ago it was generally understood that, as far as the civilized world is concerned, syphilis is one of the few new diseases that have been introduced since ancient times. It was understood that the disease was contracted by the sailors of Columbus from the Indians of the New World, carried back into Spain and Portugal from where it rapidly spread to the Spanish soldiers who, as mercenaries, introduced it into the armies of Charles VIII of France and by which it was carried into Italy as far as Naples. The army, victorious against the Neapolitans and others in their pathway succumbed to the disease, retreated into France and there was disbanded, to send syphilis home to all of the countries of Europe which had contributed to the mighty army of Charles. The Italians called it the Spanish disease, the French called it the Italian disease, the English, Germans and Turks called it the French disease and the Russians called it the Polish disease. The Spanish, alone, called it the disease of Haiti.

Aside from the time relationship, epidemiologic considerations pointed to an infection of a previously uninvaded multitude of hosts whose lack of acquired immunity was chiefly responsible for the extreme virulence of the epidemic.

But in 1913 Karl Sudhoff presented his evidence that syphilis had existed in Europe prior to Columbus' discovery of America. This opened the question anew with the result that some evidence was collected through several years purporting to show that syphilis was an ancient disease in Europe, even that the ancient Egyptians had suffered from syphilitic osteitis, as evidenced by lesions found in mummies. Probably it is well that the question was raised by an authority such as Sudhoff, since this necessitated a reexamination of the records and has resulted in the discovery of additional facts far more convincing than those previously available. The evidence for and against importation from the New World has been collected and presented by Pusey in a most interesting manner in his Gehrmann Lectures before the University of Illinois.[1]

In Germany in 1495 syphilis was often mentioned as the "never-before-seen-and-heard-of-disease." The earliest known German state document on syphilis was published in 1495, well after the time of Columbus' return and the introduction of syphilis into Germany.

Oviedo, who was in Barcelona at the time of Columbus' return and knew him and members of his crew, described how the disease was contracted from Indian women by Spaniards with Columbus and how it was brought by them to Spain. Las Casas, whose father accompanied Columbus on his second voyage, and who, himself, lived in Haiti for many years remarked "there were and still are two things which at the beginning were very dangerous to the Spaniards. One was the disease syphilis, which in Italy is known as the French malady." He, himself, describes seeing the first cases in Seville among Columbus' sailors. "It is a thing well verified that all the incontinent Spaniards that did not have the virtue of chastity on this island were contaminated."

Dias de Isla, who was a physician at Lisbon at the time of Columbus' return, described the new disease as it occurred among Columbus' sailors and later in the general population. The first and second editions of his work have been discovered and what is more interesting, the original manuscript. The original manuscript contains certain paragraphs on syphilis which were omitted from the printed work. This may have been for patriotic reasons. Dias de Isla, practicing in Barcelona in 1493, saw the very entrance of syphilis into the Old World. He states definitely that the disease was unknown before the year 1493, that it was brought by the crew of Columbus on their return from their first voyage, that a majority of the crew were infected, and that he himself treated a number of the sailors. "At the time that Admiral don Xristoual Colon arrived in Spain the Catholic Sovereigns were in the city of Barcelona. And when they went to give an account of their voyage and of what they had discovered, immediately the city began to be infected and the aforesaid disease spread."

It has been claimed that bone lesions have been found indicative of syphilis in human beings who died in the Old World long before the discovery of

America. Among others, Williams of Buffalo has made extensive investigations into this. The only incontrovertibly characteristic bone lesion is in the flat bones of the skull. Lesions of long bones might be due to other diseases. No incontrovertible evidence of syphilis of the bone has ever been presented in any skull prior to the time of Columbus except in the New World, where such evidence is relatively abundant. The search therefor has not been haphazard, as is indicated by the report of Elliot Smith based upon the examination of the mummies of 30,000 Egyptians and Nubians.

There is no definite evidence, either from morphologic studies, from historical documents, from medical treatises, or from legal enactments, of the presence of syphilis in Europe prior to the discovery of America. And, as Pusey pointedly remarks, there is no evidence in the prurient literature of the ancients. What fun Boccaccio or the authors of the Arabian Nights would have had in describing the wages of sin did they know of the ravages of syphilis!

But, after the return of Columbus it required but a short time for the disease to spread throughout the known world. The Portuguese explorers lost no time. In India the disease was recognized in 1498, after the arrival of Vasco de Gama who had left Portugal one year earlier. It appeared in Canton, China, in 1505. In Japan it was first described in 1569 when it was imported either by Chinese or Portuguese sailors.

The discovery of a New World gave birth to many new and perplexing problems, not the least among which was that of syphilis.

REFERENCE

1. Pusey, Wm. Allen: The History and Epidemiology of Syphilis, Springfield, Ill., 1933, Arthur C. Thomas.

—*W. T. V.*

CORRESPONDENCE

Remarks supplementary to editorial on "Bilirubinemia and Pneumonia" by R. A. K. in Journal of Laboratory and Clinical Medicine 18: 1197, 1933

To the Editor:

In order to establish a clear understanding of the significance of the jaundice of lobar pneumonia as it may be seen in its present stage of analysis, the following summary is submitted. This summary represents but a single phase of a concept-totality with a number of ramifications involving jaundice as a whole and its mechanism in any other clinical entity with which it may be associated. Here, however, we are concerned specifically only with the events occurring when jaundice is associated with a disease caused by a bile-soluble organism, the pneumococcus.

1. Icterus, manifest in one or all of three tests performed daily during the acute course of lobar pneumonia, has been found to be practically always present. These tests consist of the icterus index, the aqueous (direct) van den Bergh reaction, and the quantitative serum bilirubin determination, based on van den Bergh's application of Ehrlich's diazo reaction as recommended by Pröscher.

2. The visibility of icterus in the skin or sclerae is of no significance other than that visible icterus is quite characteristic of lobar pneumonia, its detection being largely a question of the visual acuity of the observer. The fundamental data must be obtained from studies on the blood serum. Numerous observations have demonstrated that no threshold for the visibility of icterus can be postulated, since visibility is dependent on qualitative as well as quantitative factors in jaundice.

3. If, regardless of other methods for characterizing the course of lobar pneumonia, cases are classified on the basis of the presence or absence of pleural fluid (not fibrin alone), demonstrable by x-ray, daily physical examination, or, preferably, chest tap, then the following conclusions are admissible:

a. Approximately 70 per cent of hospitalized patients with lobar pneumonia in this series developed pleural fluid, and in this group the mortality was approximately 15 per cent.

b. In the remaining 30 per cent of these patients fluid was not demonstrable in any way ("dry chest" type), and in this group the mortality lay between 75 per cent and 80 per cent.

A figure for the incidence of pleural fluid in pneumonia, closely approximating this finding, is reported by G. Andral, Clinique médicale, Paris, Gabon et Cie., 1824, 2, 242, and by R. T. H. Laennec, Traité de l'auscultation, Paris, G. S. Chaudé, ed. 2, 1826. It is on the basis of the presence or absence of this fluid that the interpretation of the changes in the jaundice of lobar pneumonia is made, so far as they are related to the outcome.

4. In the white race, in the absence of pleural fluid, the mortality has thus far been 100 per cent unless the icterus index exceeded 16.6. Most white patients whose icterus index curves exceeded 16.6 exhibited the downward turn in the zone 18.7 to 30, and thus far none have died whose maximum index lay in this zone whether fluid was present or absent. When, as occasionally happened, the icterus index of a white patient exceeded 30, recovery then took place only if the icterus index 100 was attained. In the negro, on the other hand, in the absence of fluid, the mortality was 100 per cent unless the icterus index exceeded 100. This is why apparently white patients are said to show negroid reactions if their icterus index curves exceed 30. True crisis is regarded as the favorable termination of the disease when fluid is absent. It is rare.

5. Upon the development of pleural fluid the rising icterus index curve is broken, and the curve tends to fall back to normal. Frequently a patient is seen for the first time after this has already occurred, and the icterus index is found to be normal. Jaundice is then

demonstrable only by the van den Bergh reaction, or in an elevation of the quantitative serum bilirubin. These patients die with direct positive van den Bergh reactions, and resolution is not assured until the van den Bergh reaction (by the ring test) becomes negative. In all cases in which data were available to show that, in the presence of fluid, the icterus index curve turned in the zone 11 to 15, thus far there have been no fatal terminations in the white race.

6. The icterus index 16.6 warrants recognition as the "Death Index" in the white race in the absence of fluid, for it must be passed if recovery is to be expected. It may be regarded as a biologic constant in the white race, and of no significance in the negro. The data suggest further that the icterus index 10, apparently of significance in the white race in conjunction with the formation of pleural fluid, is worthy of careful consideration as another possible constant.

In this entire study of icterus, as it occurs in pneumonia and other conditions, it has become apparent that many commonly accepted theories on which classifications of jaundice are based are not acceptable as working hypotheses, hence it is advisable not to attempt to classify the icterus of lobar pneumonia at the present time.

The study of the pneumonic icterus, as well as all icterus, is extraordinarily fascinating, but like all research, requires that the tests be performed by the research worker himself rather than being entrusted to a technician, unless the technician has time and interest to devote to the work specifically. It is further advisable that a single individual conduct the tests, so that the factor of error will be a constant.

In pneumonia, induced by a specific organism, it does not follow that only pneumococcus pneumonias exhibit icterus. It does follow, however, that in pneumonias caused by Friedländer's bacillus, staphylococci, and the hemolytic streptococcus, the interpretation of the movements of the icterus is wholly invalidated, for such organisms are not bile soluble. Blood cultures, x-ray examinations of the chest, sputum cultures, chest and lung punctures and cultures, and autopsies have on several occasions disproved apparent exceptions to the findings described above, especially when erroneous clinical diagnoses were made, or when the invading organism was other than the pneumococcus.

Pleural fluid is generally most conspicuous in patients approaching a fatal outcome in its presence, frequently being very transient in those that recover uneventfully, but is readily demonstrable by timely chest-tap. It may be differentiated from true empyema in that, although it is a cellular fluid, the supernatant is bright yellow as contrasted with the more or less colorless supernatants of empyema, and its cells, as exhibited by supravital staining technic, comprise a relatively high percentage of living macrophages, in contrast with the dead polymorphonuclears of empyema.

NORMAN W. ELTON, M.D.

READING, PA, SEPTEMBER 5, 1933.